UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

Departamento de Producción Animal

MECANISMOS GENÉTICOS Y ESTRATEGIAS ADAPTATIVAS DE PRODUCTORES PRIMARIOS (MICROALGAS Y CIANOBACTERIAS) EN UN ESCENARIO DE CAMBIO GLOBAL

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

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Bajo la dirección de los doctores
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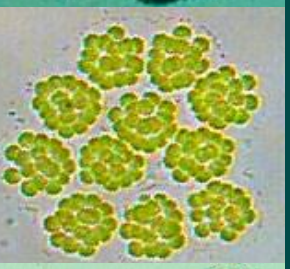

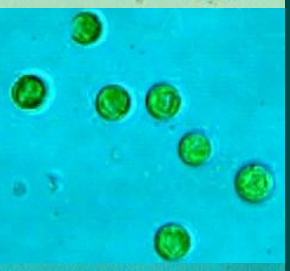
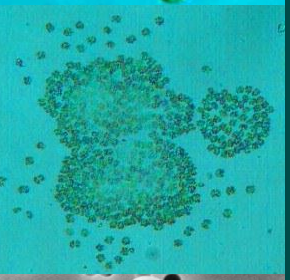
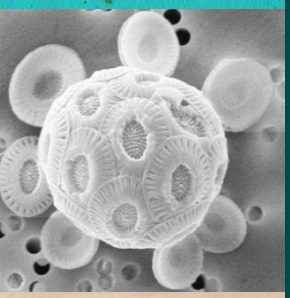

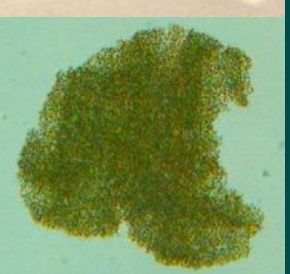

Universidad Complutense de Madrid
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Mecanismos genéticos y estrategias adaptativas de productores primarios (microalgas y cianobacterias) en un escenario de cambio global

TESIS DOCTORAL

Mónica Rouco Molina

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INFORMAN:

Que el trabajo de Tesis titulado: "**Mecanismos genéticos y estrategias adaptativas de productores primarios (Microalgas y cianobacterias) en un escenario de cambio global**", ha sido realizado en el Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid bajo nuestra Dirección.

Y para que así conste lo firmamos en Madrid a veintiséis de Enero de dos mil once.

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Mecanismos genéticos y estrategias adaptativas de productores primarios (microalgas y cianobacterias) en un escenario de cambio global

Memoria presentada por
Mónica Rouco Molina
para optar al grado de Doctor
con Mención europea

A mis padres

Con cierto temblor, el caballero arrojó todo su peso contra la vieja puerta oxidada. Había atravesado numerosas dependencias del torreón hasta llegar a este último obstáculo en una búsqueda que había durado desde que él era capaz de recordar. Detrás de esa puerta, le dijo la voz que le acompañaba (…), se encuentran muchas de las respuestas que había estado buscando (…). Se quedó sorprendido cuando la puerta se abrió fácilmente, revelando una cámara gigantesca y poco iluminada. Se trataba de una biblioteca, conteniendo legajos almacenados en estantes, que se extendían hacia el fondo, más allá de lo que su vista era capaz de alcanzar. (…). Finalmente, tomó uno de los legajos y lo llevó hasta la mesa. (…). Cuando lo abrió, descubrió que contenía caracteres para él desconocidos. (…). Estaba lleno de términos que no era capaz de entender con referencias a otros legajos de los que no se daba la localización. El caballero gritó con ira: "¿Dónde están las respuestas que se me prometieron?". "En los legajos, por supuesto", le respondió la voz casi de inmediato. "Pero entonces, ¿qué legajo debo leer?" preguntó el caballero desesperado. "Todos ellos, todos ellos", fue la respuesta. Entonces el caballero se dio cuenta de golpe de que la cualidad que se iba a ver sometida a prueba no era ni su rapidez ni su valentía, sino su paciencia. Contempló de nuevo la inmensa sala, anhelando poder enfrentarse con un dragón. La única respuesta fue un silencio intemporal. Con un profundo suspiro se quitó la armadura, la dejó en una esquina junto a sus armas y, tras sentarse lo más cómodamente que pudo, comenzó a descifrar el primer legajo.

(Robert Shapiro. Introducción de su libro: La impronta humana)

Í N D I C E

Agradecimientos

I. RESUMEN

Los ecosistemas acuáticos se están viendo fuertemente amenazados por distintas alteraciones acontecidas en un escenario de Cambio Global. Aunque aún existen numerosas incertidumbres sobre el escenario de evolución concreto, se dispone de información precisa que apoya de forma sólida y concluyente las alteraciones que se están experimentando. Entre estas alteraciones se incluye el Cambio climático y la liberación masiva de sustancias contaminantes al medio, derivados de la actividad humana. Una de las consecuencias más relevantes e inmediatas del Cambio Global es la afectación de la biota, entre la que se incluyen los organismos fitoplanctónicos, de relevante papel ecológico. Las cianobacterias y microalgas son los productores primarios de los sistemas acuáticos y por tanto la base de la red trófica en estos. Asimismo, juegan un papel esencial en los ciclos biogeoquímicos siendo una pieza clave para el mantenimiento del planeta. Por ello, la capacidad de respuesta de estos organismos ante los cambios ambientales que se auguran resulta de gran importancia.

Los trabajos incluidos en esta tesis pretenden ampliar el conocimiento sobre los mecanismos que permiten la adaptación de estos microorganismos a determinadas condiciones extremas. Para ello se estudió la respuesta ante distintos contaminantes de origen antropogénico (formol, cloranfenicol, cromo, simazina y diquat) y la exposición a diversos ambientes extremos (*Aguas Agrias, Vulcano*, y *Agrio Argentino*). Los resultados obtenidos por medio del análisis de fluctuación indican que el fitoplancton es capaz de adaptarse a la exposición a sustancias tóxicas por medio de la selección de mutantes resistentes que aparecieron al azar, antes de la presencia del agente selectivo. En el caso de la adaptación a ambientes extremos, el gradiente de toxicidad impuso distintas estrategias adaptativas para los organismos mesófilos explicando así la variación en la diversidad de especies encontrada en estos ambientes. Además de los mecanismos implicados en la adaptación, gracias a la herramienta experimental del Ratchet, se observó que el fitoplancton presenta una capacidad de adaptación diferente dependiendo tanto del grupo taxonómico y de su lugar de aislamiento. Por ello, el cambio global conducirá a alteraciones en la dinámica y estructura de las poblaciones.

Pero la adaptación no es la única fuerza conduciendo el cambio evolutivo. El estudio de la evolución de la cianobacteria Microcystis aeruginosa ante un escenario de aumento de temperatura y nutrientes se demostró que la adaptación es el principal componente conduciendo el cambio evolutivo de la tasa de crecimiento en este organismo. Sin embargo, la evolución de la producción de toxina está determinada principalmente por mecanismos de azar y contingencia histórica.

En último lugar, se estudió el efecto de la acidificación oceánica y la disponibilidad de nutrientes sobre la fisiología del cocolitofórido Emiliania huxleyi, considerando su papel importante en la regulación del ciclo del carbono. Los resultados demuestran que el proceso de calcificación se ve altamente alterado con el aumento del CO_2 atmosférico y su respuesta varía en función de la disponibilidad de nutrientes. La capacidad de asimilación de nutrientes de esta especie también se ve alterada como puede observarse por la alteración en la actividad de la enzima nitrato reductasa y alcalina fosfatasa.

I. SUMMARY

Aquatic ecosystems are under threat from alterations to their environment due to global change. Although there are still numerous uncertainties in relation to the evolution of such changes, evidence is rapidly accumulating that supports the reality of these alterations. Climate change and the release of contaminants to the environment, derived from anthropogenic activities, are fundamental parts of global change. One of the most important consequences of global change is the impact on living organisms, with marine phytoplankton being particularly affected. Cyanobacteria and microalgae play an important ecological role as the primary producers and the base of the trophic web in aquatic ecosystems. They also participate in the control of biochemical cycles, a key factor in preserving the equilibrium of the Planet. Therefore, the capacity for these organisms to respond to the predicted environmental changes is a matter of concern.

The studies presented in this thesis aim to improve our knowledge about the mechanisms allowing phytoplankton adaptation to different extreme conditions. To this end, the response of these organisms to different contaminants (formaldehyde, chloramphenicol, crome, simazime and diquat) as well as to different extreme environments (*Aguas Agrias, Vulcano*, y *Agrio Argentino*) was studied. The results, by means of the fluctuation analysis, indicate that the phytoplankton can adapt to toxic substances exposure through the selection of resistant mutants that arose randomly prior to the toxic exposure. In the case of the adaptation to extreme environments, mesophilic phytoplankton along environmental gradients showed different strategies of adaptation. We could then explain the variation in the species diversity found in these environments. In addition, by means of Ratchet protocol, we observed that phytoplankton present different capacities to adapt depending on their taxonomic group as well as their preferred habitat. Hence, global change will lead to alterations in the dynamic and structure of populations.

But adaptation is not the only force driving evolutionary change. Another components can also contribute to evolutionary process. The study of the evolution of

the cyanobacteria *Microcystis aeruginosa,* under a scenario of increasing temperatura and nutrient concentration, revealed that adaptation was the main component driving growth rate evolution, although chance events also contributed to a lesser extent. However, the evolution of toxin production was mainly determined by historic contingency and chance mechanisms.

Finally, we attempted to study the effect of ocean acidification and nutrient availability on *Emiliani huxleyi* physiology, considering its important role in the regulation of carbon cycle. The results show that calcification process is highly altered with CO_2 increase and it response varies as a function of nutrient availability. The capacity for nutrient assimilation is also altered as can be observed by the modification of nitrate reductase and alkaline phosphatase activity.

II. ESTRUCTURA GENERAL DE LA TESIS

La presente tesis expone los resultados obtenidos en distintos trabajos de investigación expuestos en forma de artículos científicos, publicados en diversas revistas contenidas en el *Science Citation Index* (SCI) o en distintas fases de publicación. Asimismo, se incluye una introducción general revisando el tema planteado y se proponen unos objetivos. Finalmente se presenta una discusión integrada de los resultados y unas conclusiones.

Los resultados se presentan divididos en 5 capítulos:

- **CAPÍTULO I. Estrategias genéticas de la adaptación rápida a ambientes extremos:**

López-Rodas,V., Marvá F., **Rouco M**, Costas, E., Flores-Moyá, A. (2008). Adaptation of the chlorophycean *Dictyosphaerium chlorelloides* to stressful acidic, mine metal – rich waters as result of pre-selective mutations. *Chemosphere*. 72: 703–707.

López-Rodas,V., Costas, E., Maneiro E., Marvá F., **Rouco, M**., Delgado, A. and Flores-Moya, A. (2009). Living in vulcan´s forge: algae adaptation to stressful geothermal ponds on Vulcano Island (S Italy) as result of pre-selective mutations. *Phycological Research* 57: 111-117.

López Rodas V., **Rouco M.,** Sánchez-Fortún,S., Flores-Moyá A., Costas E. Genetic adaptation and acclimation of phytoplankters along a stress gradient in the extreme waters of the Agrio River-Caviahue Lake system (Neuquén, Argentina) (2011). *Journal of phycology* (in press).

- **CAPÍTULO II. Estrategias genéticas de la adaptación rápida a contaminantes antropogénicos:**

López Rodas V., Perdigones N., Marvá F., **Rouco M**. and García Cabrera J.A. (2008). Adaptation of phytoplankton to novel residual materials of water pollution: An experimental model analysing the evolution of an experimental microalgae population under formaldehyde contamination. *Bulletin of Environmental Contamination & Toxicology*. 80:158–162.

Sánchez-Fortún, S., Marvá F., **Rouco M**, Costas, E., López Rodas V (2009). Toxic effect and adaptation in *Scenedesmus intermedius* to anthropogenic chloramphenicol contamination: genetic vs. physiological mechanisms to rapid acquisition of xenobiotic resistance. *Ecotoxicology* 18: 481-487.

Sánchez-Fortún, S., López-Rodas, V., Navarro, M., Marvá, F., D'ors A., **Rouco M**., Haigh-Florez D., and Costas, E. (2009) Toxicity and adaptation of Dictyosphaerium chlorelloides to extreme chromium contamination. *Environmental toxicology and chemistry* 28: 1901-1905.

Marvá F., López-Rodas V., **Rouco M**., Navarro M., Toro F.J., Costas E., Flores-Moyá A (2009). Adaptation of green microalgae to the herbicides simazine and diquat as result of pres-selective mutations. *Aquatic Toxicology*. doi: 10.1016/j.aquatox.2009.10.009.

- **CAPÍTULO III. Determinación de la capacidad diferencial de adaptación y máxima capacidad adaptativa:**

Huertas, I.E., **Rouco, M**., López-Rodas, V, Costas, E. 2010. Estimating the capability of different phytoplankton groups to adapt to contamination: herbicides will affect phytoplankton species differently. *New phytologist* 188: 478-487.

Rouco M, López-Rodas V, Huertas E, Costas E (2011) Differential capacity for adaptation of two green algae and one cyanobacteria to copper sulphate: Implications for the management of copper of water reservoirs (in preparation).

Huertas IE, **Rouco M**, López-Rodas V, Costas E. Warming will Affect Phytoplankton Differently: Evidence through a Mechanistic Approach. Proceedings of the Royal Society of London B. Biological Sciences (in press) doi: 10.1098/rspb.2011.0160.

- **CAPÍTULO IV. Contribución de las distintas fuerzas en el cambio evolutivo:**

Rouco M, López-Rodas V, Flores-Moyá A, Costas E (2011) Evolutionary changes in growth rate and toxin production in the cyanobacterium *Microcystis aeruginosa* under a scenario of eutrophication and temperature increase. Microbial ecology (in press. DOI: 10.1007/s00248-011-9804-0).

- **CAPÍTULO V. Efecto de la disponibilidad de nutrientes bajo distintos escenarios de CO_2:**

Rouco M, Branson O, Lebrato M, Iglesias-Rodríguez D. Effect of nitrate and phosphate availability on *Emiliania huxleyi* physiology under different CO_2 scenarios (in preparation).

III. INTRODUCCIÓN

"El experimentador que no sabe lo que está buscando
no comprenderá lo que encuentra"

(Claude Bernard. Fisiólogo francés)

➢ IMPORTANCIA DE LOS SISTEMAS ACUÁTICOS.

Los sistemas acuáticos ejercen funciones importantes para el correcto funcionamiento del planeta. Entre ellas destacan su papel fundamental en el mantenimiento de los ciclos naturales de nutrientes (ciclo del carbono, nitrógeno, fósforo, etc.) así como sustento para fauna y flora. Entre los dos tipos principales de ecosistemas acuáticos se encuentran el ecosistema marino y de agua dulce. Los océanos cubren aproximadamente tres cuartas partes de la superficie terrestre y contienen cerca del 97 % del agua del planeta (Barange *et al.* 2010). Se ha estimado que alrededor del 80 % de la vida en la Tierra depende de la *salud* de océanos (Barange *et al.* 2010). Los ecosistemas de agua dulce cubren tan solo un 0.8 % de la superficie de la Tierra conteniendo únicamente el 0.01 % del agua del planeta (McAllister *et al.* 1997), pero albergan una gran variedad de especies de organismos vivos. Se ha estimado que el 40% de la diversidad global de peces y un cuarto de la de vertebrados habita en ecosistemas de agua dulce (Lundberg *et al.* 2000). Los ecosistemas acuáticos no solo son importantes por la gran cantidad de organismos que habitan. Muchos organismos terrestres, entre ellos incluido el hombre dependen de este importante recurso para su supervivencia. La preservación de los sistemas acuáticos, por tanto, es importante para la conservación de la vida y diversidad en el planeta.

➢ IMPORTANCIA ECOLÓGICA DEL FITOPLANCTON

El fitoplancton es el componente autótrofo de la comunidad planctónica. Engloba organismos fotosintéticos procariotas (cianobacterias) y eucariotas (microalgas) que habitan cerca de la superficie de la columna de agua donde son capaces de captar la luz necesaria para sustentar la fotosíntesis.

Estos organismos son importantes para el sustento de la vida en la Tierra. Por un lado, el fitoplancton es el responsable de la mayor parte del oxigeno presente en la atmósfera terrestre liberado al medio mediante el proceso de la fotosíntesis. Asimismo, la fijación de materia orgánica, conocido como producción primaria, es la base de la cadena trófica en los sistemas acuáticos, tanto marinos como de agua dulce, sosteniendo

la vida en éstos. El 45 % de la fotosíntesis ocurre en ecosistemas acuáticos (Falkowski 1994, Field *et al.* 1998) siendo el fitoplancton oceánico el responsable de gran parte de la producción primaria neta global (Valiela 1995; Behrenfeld *et al.* 2001). La fotosíntesis sustenta la mayor parte de la vida en la Tierra y por tanto es la base de la biomasa y biodiversidad en el planeta. La producción neta global en los océanos es de aproximadamente 50 Pg C por año producidos por una biomasa de 1 Pg C, lo que corresponde a una fracción de tan solo el 0.2 % de la biomasa fotosintéticamente activa de la Tierra (revisado por Falkowski *et al.* 1998). Esto significa, que la renovación del fitoplancton es rápida y cualquier factor que afecte a esta capacidad de renovación derivará finalmente en la alteración de ambos procesos.

Por otro lado, estos organismos son claves en el mantenimiento del equilibrio planetario y su clima a través del intercambio entre atmósfera y océano de gases relevantes en el mantenimiento climático (Rost *et al.* 2008). El fitoplancton es un componente indispensable del ciclo biogeoquímico de distintos nutrientes como son el nitrógeno, fósforo o carbono.

La abundancia del plancton y su distribución depende de factores ambientales como concentración de nutrientes, estado físico de la columna de agua, así como la abundancia de otros organismos planctónicos o predadores. La irradiancia y la temperatura son variables ambientales importantes controlando las tasas de fotosíntesis. Por ello, la producción primaria será ampliamente afectada por fenómenos de cambio ambiental tanto de origen natural como aquellos derivados de la actividad antropogénica.

➢ CICLO DEL CARBONO EN EL OCÉANO

El ciclo del carbono es un ciclo biogeoquímico por el cual el carbono es intercambiado entre hidrosfera, biosfera, geosfera y atmósfera de la Tierra. El ciclo del carbono en el océano implica procesos tanto físicos, químicos, geológicos como biológicos dando lugar a continuos flujos de intercambio. Incluye la transferencia de CO_2 desde la atmosfera hasta el océano, la fijación del carbono por el fitoplancton, el

flujo del carbono a través de la cadena trófica, así como la deposición y enterramiento del mismo en el océano profundo por medio de distintos mecanismos.

Las algas y organismos fotosintéticos acuáticos participan en una parte importante del ciclo del carbono. Modifican el sistema carbonato así como el pH del agua de forma natural mediante el desarrollo de su metabolismo: fotosíntesis y respiración (Hurd *et al.* 2009). La producción de carbono orgánico por el fitoplancton mediante el proceso de la fotosíntesis da lugar a la reducción de la concentración de CO_2 en la superficie oceánica (Fig. 1.a) actuando como "sumidero" para el CO_2 así como dando lugar a un aumento del pH.

Fotosíntesis: $6CO_2 + 12H_2O \rightarrow C_6H_{12}O_6 + 6O_2 + H_2O$ **(a)**

Respiración: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ **(b)**

Calcification: $Ca^{2+} + 2\ HCO_3^- \rightarrow CaCO_3^- + CO_2 + H_2O$ **(c)**

Fig. 1. Ecuaciones generalizadas de los procesos de fotosíntesis (a), respiración (b) y calcificación (c).

El secuestro del carbono en el océano profundo forma asimismo parte activamente en la reducción del carbono en aguas superficiales. La magnitud del secuestro de carbono depende en gran medida de la disponibilidad de macronutrientes en la superficie oceánica así como de alteraciones en "Redfield ratio" C:N:P (106:16:1). Un exceso en el consumo de carbono por parte del fitoplancton debido a alteraciones en la tasa Carbono: Nitrógeno (C:N), fundamentalmente durante el desarrollo de blooms fitoplanctónicos, puede dar lugar a la liberación y acumulación temporal de materia orgánica disuelta rica en carbono (DOC) (Kähler y Koebe 2001) que constituye una fuente de carbono disuelto en aguas superficiales. En la mayoría de los casos, esta materia presenta naturaleza fundamentalmente polisacárida y ácida (Benner 2002) lo que le da una consistencia pegajosa con tendencia a coagular dando lugar a material particulado conocido como TEP (partículas exopoliméricas transparentes) (Passow 2002; Engel 2002, Engel *et al.* 2004b). Estos compuestos particulados forman parte en los procesos de formación de "nieve marina" (compuesta principalmente de materia

orgánica viva o muerta, procedente de animales y plantas, materia fecal y arena) contribuyendo a la exportación del carbono orgánico e inorgánico de la superficie al océano profundo (Passow 2001; Passow *et al.* 2002). Este carbono en aguas profundas puede ser asimismo remineralizado por el metabolismo microbiano, contribuyendo al enriquecimiento del océano interior, o enterrado en sedimentos. La asimilación de carbono inorgánico disuelto (DIC) durante la fotosíntesis así como la sedimentación en el océano profundo a través de la formación de TEPs, organismos muertos o material fecal contribuye a la reducción del CO_2 en la superficie oceánica considerando de este modo al océano como "Bomba biológica" sumidero de CO_2. Este mecanismo, conocido como "bomba biológica" ayuda al mantenimiento de la concentración atmosférica de CO2 unos 400 ppm por debajo de lo que sería si no tuviera lugar el proceso fotosintético (Falkowski 1997).

Por el contrario, el proceso respiratorio y calcificación (Fig. 1 b y c) son considerados como "fuentes" potenciales de CO_2 a la atmósfera (Zondervan 2007; Hurd *et al.* 2009). La incorporación de carbono inorgánico en forma de carbonato cálcico para la formación de las estructuras calcáreas en diversos organismos (proceso de calcificación) contribuye a la reducción de la alcalinidad del medio reduciendo la capacidad del océano de secuestro de CO_2 atmosférico.

Por este motivo, la tasa calcificación: fotosíntesis (PIC:POC o carbono particulado inorgánico incorporado mediante el proceso de calcificación: carbono particulado orgánico incorporado en el proceso de fotosíntesis) se considera un parámetro biogeoquímico clave para la determinación del flujo neto de carbono entre la atmósfera y el océano (Frankignoulle *et al.* 1994; Buitenhius *et al.* 2001; Conservan 2007).

➢ EL PROBLEMA: CAMBIO CLIMÁTICO Y LA ACTIVIDAD HUMANA

"If today is a typical day on planet Earth, we will lose 116 square miles of rainforest, or about an acre a second. We will lose another 72 square miles to encroaching deserts, as a result of human mismanagement and overpopulation. We will lose 40 to 100 species, and no one knows whether the number is 40 or 100. Today the human population will increase by 250,000. And today we will add 2,700 tons of chlorofluorocarbons to the atmosphere and 15 million tons of carbon. Tonight the Earth will be a little hotter, its waters more acidic and the fabric of life more threadbare."

(David Orr (1991) "What is Education For?"
http://www.context.org/ICLIB/IC27/Orr.htm.)

"We *are in a pronounced early stage of an extinction event that would probably be, by the end of this century if human activities continue unabated (...)"*

(Edward O. Wilson. Ganador de dos premios Pulitzer)

La historia de la Tierra cuenta con varias extinciones en el transcurso de su evolución. De hecho se calcula que el 99% de las especies que un día poblaron la Tierra han desaparecido (Blaschke 2007). La Tierra se ve sumida de forma natural en un ciclo de periodos glaciales e interglaciares (Hays *et al.* 1976) y en la actualidad, nos encontramos sumidos en una pausa interglaciar que ha dado una oportunidad a la vida en la Tierra tal y como hoy la conocemos. Sin embargo, la invasión humana de los hábitats naturales así como la contaminación masiva de agua, tierra y atmósfera está provocando la aceleración del cambio evolutivo de las especies (Palumbi 2001; Myers y Knoll 2001) derivando en una aumento de la tasa media de extinción (Woodruff 2001). Ya no es duda que nos encontramos ante "la sexta extinción". La crisis de la biodiversidad es real. Muchos se plantean si el cambio climático proviene únicamente de un episodio normal dentro de los ciclos naturales terrestres. Lo que sí es cierto es que

la actividad humana está contribuyendo ampliamente a la aceleración del proceso (Pimm *et al.* 1995; Vitousek *et al.* 1996¸ Vitousek *et al.* 1997).

Entre las consecuencias de la actividad humana seleccionadas en esta tesis para su estudio como agentes selectivos se encuentran:

- Liberación de residuos procedentes de actividades agrícolas, industriales o urbanas.
- Eutrofización.
- Aumento de las concentraciones atmosféricas de CO_2.
- Aumento de la temperatura.
- Alteración en la disponibilidad de nutrientes.

Liberación de residuos procedentes de actividades agrícolas, industriales o urbanas

La producción y emisión de contaminantes al medio acuático deriva distintas actividades humanas: núcleos de asentamientos urbanos, desarrollo de infraestructuras o construcción, actividades agrícolas, desarrollo industrial, turismo, etc. Entre los contaminantes más destacados se incluyen: fertilizantes, pesticidas y agroquímicos; residuos domésticos y municipales y aguas residuales; aceites, metales pesados y elementos traza; compuestos orgánicos y contaminación biológica; plásticos (revisado por Islam y Tanaka 2004). Como ejemplo, solo una pequeña fracción del herbicida aplicado en actividades de agricultura intensiva, menor de un 0.1 %, alcanza su diana, mientras el gran porcentaje restante se elimina al medio contaminando tanto suelo, como agua y aire (Arias-Estévez *et al.* 2008) a través de fenómenos de escorrentía o filtrado (Beck *et al.* 1993). Por ello, no es sorprendente que se observen un gran número de herbicidas como residuos en aguas del norte de América y europeas (Kreuger 1998; Battaglin *et al.* 2000; Scribner *et al.* 2000). Asimismo, los herbicidas aplicados al terreno interactúan tanto con la fracción orgánica como inorgánica. Estas interacciones pueden provocar la disociación del herbicida en distintas fases influenciando su comportamiento tanto a corto como a largo plazo (Sheng *et al.* 2001). Algunas de estas sustancias, como los metales pesados son necesarias para el correcto desarrollo de

distintos procesos metabólicos del fitoplancton. Sin embargo, su presencia en exceso, derivado fundamentalmente de actividades humanas, provoca efectos tóxicos en plantas y animales (Rai *et al.* 1981). La presencia de estas sustancias tóxicas extrañas (ya sea por su escaso contacto previo con el organismo o por su naturaleza de nueva síntesis) en el medio acuático presenta, por tanto, un nuevo reto adaptativo para la mayoría de los organismos en función de la naturaleza, toxicidad y dosis que la sustancia alcanza en el medio. La respuesta de estos microorganismos depende asimismo de la sensibilidad de cada especie. Dependiendo de la sensibilidad diferencial, la interacción competitiva podría dar lugar a la sustitución de algas susceptibles por algas resistentes afectando de tal manera a la estructura de la comunidad acuática concreta (Kasai y Hanazato 1995; Bérard *et al.* 1999).

Eutrofización

El término de eutrofización designa el aporte más o menos masivo de nutrientes inorgánicos en un ecosistema acuático. Podría ser incluida dentro del apartado anterior puesto que el aporte masivo deriva de la descarga de aguas residuales no tratadas ricas fundamentalmente en nitrógeno, procedentes de actividades urbanas, industriales y agrícolas. La eutrofización es una de las causas generadoras de cambios en la composición de especies, estructura y función de los ecosistemas acuáticos (Islam y Tanaka 2004) derivado tanto del aporte masivo de nutrientes como del desequilibrio en el ratio Nitrógeno: Fósforo de las aguas (Fujimoto *et al.* 1997). Asimismo provoca aumento en la biomasa y productividad de las comunidades (Epstein *et al.* 1993; Riegman 1995). El aumento en la proliferación de blooms de fitoplancton tóxico ha sido asociado a fenómenos de eutrofización en muchos casos (Epstein *et al.* 1993; Hallegraeff 1993).

Aumento de CO_2 atmosférico. Previsiones de futuro.

El dióxido de carbono (CO_2) es un gas presente en la atmósfera que junto a otra serie de gases -vapor de agua (H_2O), metano (CH_4), óxidos de nitrógeno (NO_x), ozono (O_3) y clorofluorocarbonos (CFCs)- contribuye al fenómeno natural de efecto invernadero que mantiene una adecuada y cálida temperatura terrestre. Desde tiempos

pre-industriales la emisión global de estos gases de efecto invernadero ha aumentado (Fig. 2 Izqda.) como consecuencia de las actividades humanas registrando un aumento de hasta el 70% entre el año 1700 y 2004 (IPCC 2007), excediendo el rango natural observado durante los últimos 650.000 años. La concentración global atmosférica de CO_2 aumentó de 280 ppm en tiempos pre-industriales (antes de que el escocés James Watt inventara la máquina de vapor en 1971) hasta 379 ppm registrados en el año 2005 (IPCC 2007). El observatorio de Manua Loa en Hawai detectó una concentración de 390 ppm de CO_2 en el último registro de 2010 (ftp://ftp.cmdl.noaa.gov/ccg/co2/trends/co2_mm_mlo.txt).

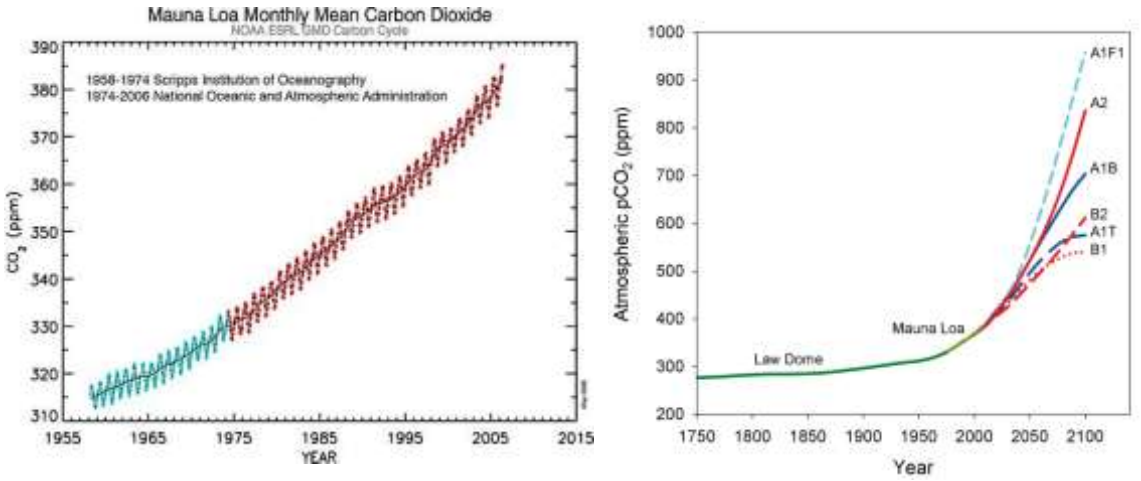
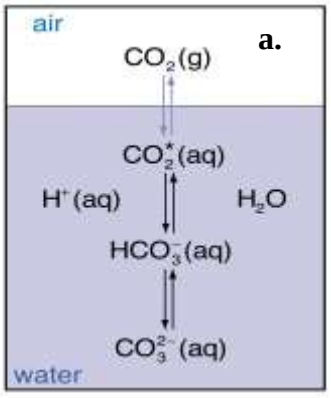


Fig. 2. Izqda. Registro temporal del aumento de dióxido de carbono (CO_2) atmosférico durante las últimas cinco décadas. Datos del observatorio atmosférico Manua Loa (Hawai). Dcha. Serie temporal de los niveles de dióxido de carbono (CO_2) atmosférico: desde 1750-1950 (datos procedentes de "ice cores" de Law Dome, en la Antártida - en verde oscuro); datos de las últimas dos décadas (observatorio atmosférico Manua Loa - en verde claro); predicciones hasta el 2100 a partir de modelos desarrollados (en rojo y azul). Fuente: Barry *et al.* 2010.

Se predice que el incremento del CO_2 atmosférico continuará durante el siguiente siglo o más allá, y las peores predicciones auguran que si las emisiones antropogénicas continúan se pueden alcanzar niveles de hasta 1000 ppm en el año 2100 (Nakicenovic y Swart 2000; Raven *et al.* 2005) (Fig. 2 Dcha.). Como otros gases, el

dióxido de carbono obedece la Ley de Henry por la que "*a una temperatura constante, la cantidad de gas disuelta en un líquido es directamente proporcional a la presión parcial que ejerce ese gas sobre el líquido*". Por ello, el aumento de la concentración de dióxido de carbono en la atmósfera conducirá a un aumento de la concentración en la superficie oceánica. Los océanos han absorbido entre un tercio (Sabine *et al.* 2004) y más de la mitad de las emisiones de CO_2 a la atmósfera desde el comienzo de la Revolución Industrial (Raven *et al.* 2005) siendo considerado el segundo sumidero mayor de CO_2 después de la atmósfera en sí (Canadell *et al.* 2007).

La disolución del CO_2 en aguas superficiales provoca un aumento de la concentración de iones hidrógeno [H^+] resultando en la reducción del pH de los océanos, fenómeno que se ha denominado "acidificación oceánica" (Fig. 3). Se ha observado una disminución de 0.1 puntos de pH de 8.2 en 1970 hasta 8.1 en el océano actual pero se espera una reducción de hasta 0.3 y 0.4 unidades en 2100 (Orr *et al.* 2005). Distintos autores sugieren que si las emisiones no se restringen se podría alcanzar un pH menor de 7.5 en el año 2300 (Caldeira y Wickett 2003). Distintos registros en la Serie temporal oceánica ESTOC establecida en aguas cercanas a las Islas Canarias en 1994, mostraron una disminución anual del pH de -0.0017 ± 0.004 unidades de pH durante el periodo comprendido entre el año 1994 y 2004 (Santana-Casiano *et al.* 2007).



b.

$$[CO_2] + [H_2O] \leftrightarrow [H_2CO_3] \quad (1)$$

$$[H_2CO_3] \leftrightarrow \mathbf{[H^+]} + [HCO_3^-] \quad (2)$$

$$[HCO_3^-] \leftrightarrow [CO_3^{2-}] + 2\mathbf{[H]^+} \quad (3)$$

Fig. 3. Equilibrio químico del en el agua. a. Disolución del CO_2 en el agua (Fuente: Dickson 2010).b. Reacciones involucradas en la liberación de iones hidrógeno [H^+] al medio tras la disolución de CO_2 en agua de mar.

Alteración del sistema carbonato con un aumento del CO_2 atmosférico

El sistema carbonato reacciona ante cualquier alteración modificando sus componentes principales (Schulz *et al.* 2009). Como consecuencia de la disolución del CO_2 adicional en las aguas superficiales, se produce una re-equilibración del sistema carbonato (Shi *et al.* 2009) y con ello, modificación del ciclo de carbono en los océanos. El aumento de pCO_2 viene acompañado de una reducción de CO_3^{2-}, pH y Ω. (Fig. 4).

El aumento de la concentración de CO_2 disuelto está provocando y provocará importantes alteraciones en los sistemas biológicos. La mayor parte de los organismos fitoplanctónicos viven en la zona eufótica, parte de la columna de agua que recibe la luz solar suficiente para que tenga lugar el proceso de fotosíntesis. Considerando que esta zona es la que está experimentando el mayor aumento en CO_2 disuelto, la fisiología del fitoplancton se verá fundamentalmente alterada (Wu *et al.* 2008).

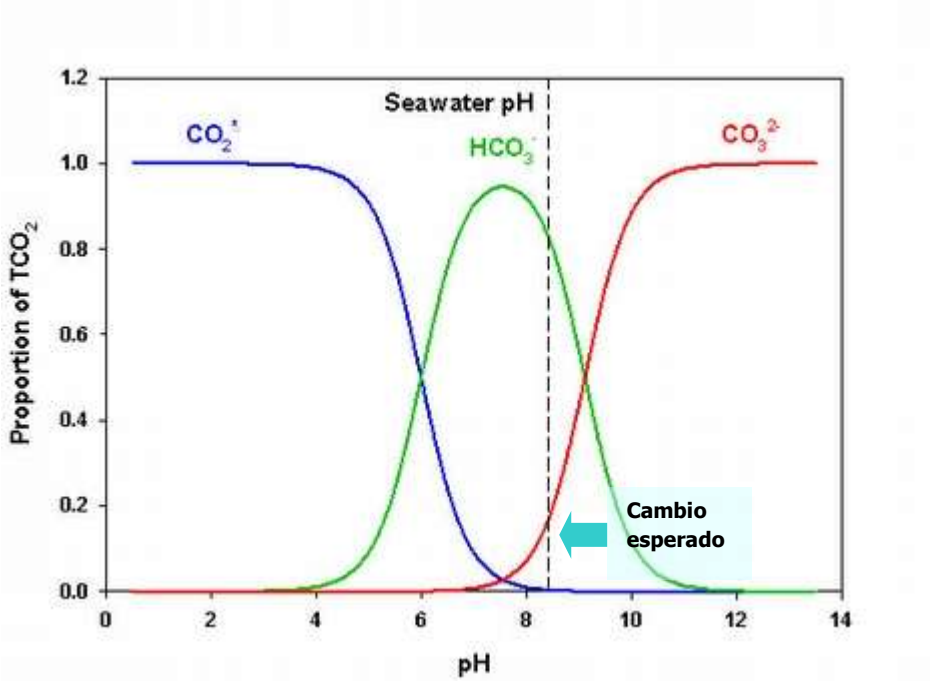


Fig. 4. Sistema carbonato en agua de mar. Representación de la proporción relativa de los distintos componentes en función del pH.

Calentamiento terrestre

Otra consecuencia del aumento de la emisión de gases invernadero y fundamentalmente CO_2 es el calentamiento terrestre. Se ha observado un aumento de la temperatura del aire de más de 0.7 ºC durante el siglo pasado (Fig. 5) derivando en aumentos de la temperatura a nivel terrestre y acuático (IPCC 2007). Entre 1995 y 2006 se registraron las temperaturas globales superficiales más altas desde 1850 (IPCC 2007). Se predice que para final del siglo XXI la temperatura de los sistemas acuáticos podría aumentar entre 1 y 7 ºC dependiendo del ecosistema acuático y los escenarios de emisión (IPCC 2007). El calentamiento está afectando en gran medida los sistemas biológicos terrestres provocando alteraciones en eventos de floración, migración de pájaros, puesta de huevos, etc. Asimismo, se espera que el aumento de la temperatura del agua dé lugar a cambios en la abundancia y distribución de especies dependiendo de su capacidad de adaptación concreta.

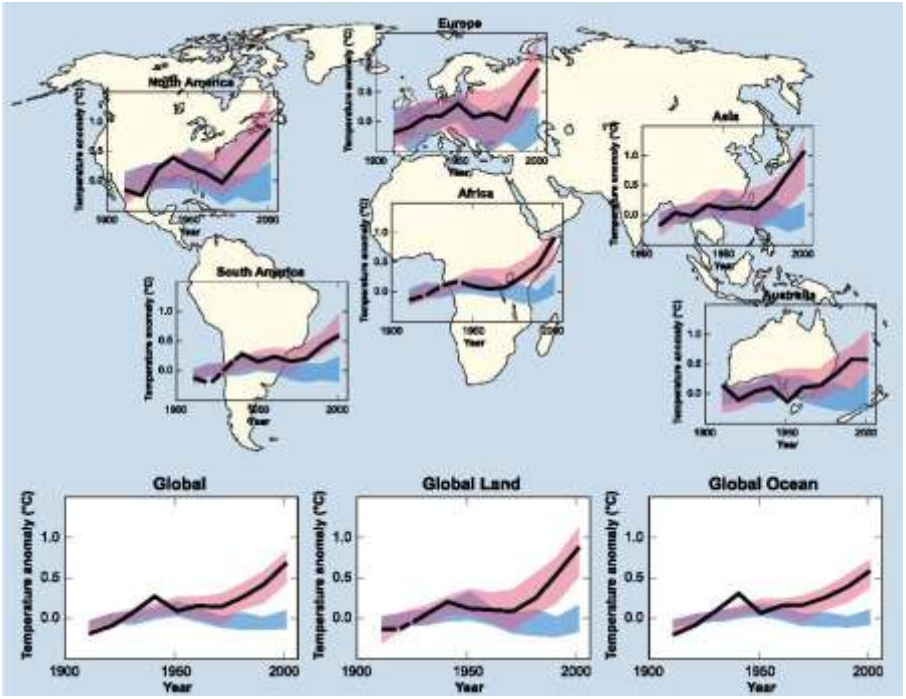


Fig. 5. Cambio en la temperatura terrestre simulado por modelos climáticos considerando solo el cambio natural (en azul) o considerando tanto el cambio natural como la influencia antropogénica (rosa). Fuente: IPCC 2007.

Diversidad en la disponibilidad de nutrientes en el océano. Alteración de los ciclos naturales

La distribución de nutrientes en el océano es muy variada. Las aguas profundas, frías y más densas, son en general más ricas en nutrientes que las aguas superficiales. Asimismo, la variación latitudinal-longitudinal es clara. Las zonas de aguas templadas, altas latitudes y sistemas de "afloramiento", reciben nutrientes de aguas profundas a través de distintos fenómenos como el transporte vertical de aguas derivado de la acción del viento y desplazamiento de capas superficiales. Sin embargo, el fitoplancton en latitudes bajas, giros subtropicales o aguas estratificadas la concentración de nutrientes depende de la recirculación a nivel local así como del flujo diapicnal de nutrientes (Cermeño *et al.* 2008). Electronic Atlas of Word Ocean Circulation Experiment (WOCE) proporciona datos de la distribución de nutrientes en el océano a nivel global (http://www.ewoce.org/) durante la década entre 1988-1998, pudiendo observar claramente la variabilidad en la distribución mencionada.

Fenómenos de cambio climático, como puede ser aumentos en la temperatura de las aguas superficiales, estratificación vertical de las aguas, así como fenómenos acentuados de viento determinarán cambios en la distribución de nutrientes en futuros océanos así como en los ciclos naturales de los mismos (Gregg *et al.* 2003; Polovina *et al.* 2008; Canfield *et al.* 2010, Cermeño *et al.* 2010).

➢ RESPUESTA DE LOS ORGANISMOS ANTE EL CAMBIO AMBIENTAL

"The diversity of the phytoplankton is explicable primary by a permanent failure to achieve equilibrium as the relevant external factors change".

(Hutchinson 1961. The paradox of the plankton)

El hábitat en el que los organismos viven no es constante. Está normalmente sometido a fluctuaciones y cambios de distinto orden derivado tanto de fenómenos naturales como de la acción del hombre. Cuando los organismos son expuestos a condiciones locales diferentes de su rango normal de fluctuación deben responder ante ese cambio si quieren asegurar su supervivencia. Entre las principales respuestas ante el cambio ambiental se encuentran: desplazamiento o escape, adaptación fisiológica (aclimatación) o adaptación genética. En último término, cuando la presión selectiva es tan elevada que no se puede asegurar ningún mecanismo de adaptación el cambio ambiental conducirá a la extinción de la especie u organismo en concreto.

La primera respuesta consiste en el desplazamiento hacia ambientes más propicios. Obviamente, esto solo puede ser asegurado en aquellos organismos que dispongan de motilidad propia. El fitoplancton, como su definición indica son "organismos fotosintéticos que flotan o nadan débilmente en el cuerpo de agua en que se encuentran". Dependen por tanto de los movimientos de corrientes de agua para su desplazamiento. Existen determinados organismos fitoplanctónicos flagelados o poseedores de vesículas de gas, como las cianobacterias, que son capaces de evitar estrés lumínico. Muestran una fototaxis positiva o negativa desplazándose hacia o contra la fuente de luz, respectivamente, en función de la intensidad lumínica (Rai y Gaur 2001). La quimiotaxis para evitar estrés químico no es muy reconocida (Rai y Gaur 2001). De cualquier manera, sería difícil la evasión de un ambiente contaminado teniendo en cuenta la motilidad de estos organismos y la extensión del agente tóxico en el medio.

Las dos siguientes respuestas dependen altamente de su rango de tolerancia fisiológica. Si las nuevas condiciones no exceden los límites de tolerancia fisiológica de

los organismos, la supervivencia puede asegurarse mediante adaptación fisiológica o aclimatación. Esto es mediante la modificación de la expresión de sus genes o componentes estructurales de genes existentes (Bradshaw y Hardwick 1989; Belfiore y Anderson 2001). Sin embargo, si las condiciones selectivas superan los límites de su tolerancia fisiológica, la supervivencia dependerá únicamente de la evolución adaptativa (Sniegowski y Lenski 1995; Sniegowski 2005). Los organismos con genotipo más resistente serán los adecuados para sobrevivir a las nuevas condiciones extremas y por tanto los ascendientes de la nueva población resistente. En el caso de los organismos con reproducción asexual, la variación a partir de la cual cuenta el proceso selectivo proviene del proceso de mutación. En este caso, las mutaciones que confieren resistencia aparecen al azar de forma espontánea antes de la presencia del agente selectivo como demostraron Luria and Delbrück en su trabajo magistral presentado en 1943. Sin embargo, años más tarde, Cairns *et al.* (1988) observaron la posibilidad de la ocurrencia de "*adaptive mutations*". En este caso, y en presencia de un agente selectivo no letal, existe la posibilidad de que los organismos experimenten mutaciones dirigidas ante la presencia del agente en concreto. Dichos trabajos reabrieron de nuevo la polémica entre aproximaciones adaptativas lamarkistas y darwinianas (Sniegowski y Lenski 1995, Roth *et al.* 2006, Roth 2010).

➢ MÁXIMA CAPACIDAD DE ADAPTACIÓN DE LOS ORGANISMOS

"Time is in fact the hero of the plot. The time with which we have to deal is of the order of two billion years. What we regard as impossible on the basis of human experience is meaningless here. Given so much time, the "impossible" becomes possible, the possible probable, and the probable virtually certain. One has only to wait: time itself performs the miracles".

(George Wald, 1954. Científico estadounidense)

Como Darwin observó ya hace más de un siglo y medio, existe una variación natural entre los individuos dentro de una especie. Esa variación permite la selección de los individuos más capacitados en un determinado ambiente dependiendo de su plasticidad y capacidad de variación genética. La fuente última de variación corresponde al fenómeno de mutación y como propusieron Sniegowski y Lenski (1995), la evolución adaptativa dependerá exclusivamente de las nuevas mutaciones que confieran resistencia. Como hemos visto en el apartado anterior, el genoma de los organismos puede experimentar mutaciones espontáneas, pudiendo alguna de ellas otorgar de cierta ventaja selectiva al organismo que las sobrelleva. En el caso de organismos con reproducción asexual, esa mutación será transmitida a la descendencia de forma directa, por lo que aquellas mutaciones beneficiosas en un ambiente concreto podrán ser mantenidas en la población en función de la intensidad de la presión selectiva así como de la ventaja selectiva de la mutación en concreto (Elena y Lenski, 2003). Pero la capacidad adaptativa de los organismos es limitada. La capacidad adaptativa de microalgas a ambientes selectivos concretos, así como la naturaleza de la mutación que confiere la resistencia, ha sido ampliamente estudiada, siendo parte de esos trabajos incluidos en esta tesis (ver capítulo 1 y 2). Sin embargo, la capacidad máxima de adaptación de estos organismos resulta asimismo interesante, considerando los fenómenos de cambio ambiental que han ocurrido y que se pronostican. Sabemos que las microalgas presentan una gran capacidad de adaptación pero, hasta donde serán capaces de resistir? serán todas las especies capaces de sobrevivir de la misma manera?

➢ CONTRIBUYENTES EN EL PROCESO EVOLUTIVO

"*All the theories engender a dark side among uncritical acolytes".*
(Stephen Jay Gould, 1991, p. 52)

''The world is full of mysteries. Life is one. The curious limitations of finite minds are another. It is not the business of an evolutionary theory to explain these mysteries. Such a theory attempts to explain events of the remote past in terms of general laws known to be true in the present, assuming that the past was no more, but no less, mysterious than the present.''

(John Burdon Sanderson Haldane, 1932)

El cambio evolutivo es un proceso complejo y dinámico sujeto a distintas fuerzas biológicas. En 1859 Darwin expuso su teoría de la evolución en su obra fundamental "El origen de las especies" en el que consideraba que la selección natural tenía un lugar de preferencia entre las fuerzas evolutivas. A pesar de la corriente extremista que tomó su nombre, que defendía la selección natural como única fuerza evolutiva, Darwin, desde un principio, aceptó la existencia de otros mecanismos involucrados en el proceso de evolución:
"*I am convinced that Natural Selection has been the main but not exclusive means of modification" (Darwin 1859 p.7)".*

Distintos autores han postulado durante años la existencia de otra serie de fuerzas que junto a la selección natural contribuyen al cambio evolutivo (Lewontin 1974; Gould y Lewontin 1979; Gould 1989; Kimura 1983; Parker y Smith 1990). Entre estas fuerzas, la contingencia histórica y el azar son consideradas contribuciones fundamentales. Dentro de azar se incluye tanto la fijación o pérdida de nuevos genes o caracteres por deriva genética como la variación originada por mutaciones al azar (Suzuki *et al.* 1989), siendo la mayor parte de éstas últimas neutrales, no sujetas a la acción de la selección (Kimura 1968, 1983). La evolución requiere variación, ya que la selección natural no puede operar sin una amplia gama de alternativas. Se ha considerado la mutación, como la fuente fundamental de variabilidad genética debido a

que solo el fenómeno de mutación es capaz de dar lugar al nacimiento de nuevos alelos (Spiess 1989; Kimura 1989). Asimismo, Gould y Lewontin propusieron el efecto de la contingencia histórica asumiendo que los resultados evolutivos son dependientes de distintos puntos ancestrales (Gould y Lewontin 1979). Como formuló Dollo (1970) en su "ley de la irreversibilidad": "*An organism is unable to return, even partially, to a previous stage already realized in the ranks of its ancestors*".

Los organismos exhiben toda una variedad de caracteres que no constituyen adaptaciones y pueden no favorecen la supervivencia de un modo directo. Existen dos principios que llevan al cambio no adaptativo (revisado por Gould 1989): a) los organismos son sistemas integrales y un cambio adaptativo en una de sus partes puede llevar a modificaciones no adaptativas de otros caracteres ("correlaciones de crecimiento" en términos de Darwin); b) un órgano construido bajo la influencia de la selección para un papel específico puede ser capaz, como consecuencia de su estructura, de realizar también otras muchas funciones no seleccionadas.

El papel de las distintas fuerzas que contribuyen al proceso evolutivo ha sido ampliamente debatido. Sin embargo, no se han desarrollado muchos experimentos para evaluarlo. En esta tesis, en el capítulo 4 en concreto, se presenta un diseño experimental modificado a partir de aquel propuesto por Travisano *et al.* (1995) en el que se estudia la contribución de tres componentes en el proceso evolutivo: Adaptación, azar e historia.

IV. OBJETIVOS

1. Analizar la capacidad de adaptación del fitoplancton a ambientes naturales extremos estudiando los distintos mecanismos que permiten el proceso de adaptación así como las tasas de mutación que pueden permitir este proceso de adaptación.

2. Estudiar la capacidad genética de adaptación del fitoplancton a la exposición a contaminantes de origen antropogénico: herbicidas, antibióticos, metales pesados y distintos biocidas, caracterizando tanto la tasa de mutación como su naturaleza.

3. Determinar la capacidad diferencial de adaptación y la máxima capacidad de adaptación del fitoplancton ante distintas situaciones extremas: aumento de temperatura y exposición a contaminantes ambientales.

4. Estudiar la contribución relativa de distintas fuerzas evolutivas (adaptación genética, azar y contingencia histórica) en el proceso de cambio de tasa de crecimiento y producción de toxina en la cianobacteria *Microcystis aeruginosa* cuando es expuesta a distintas condiciones de cambio ambiental.

5. Investigar la influencia de la acidificación oceánica y disponibilidad de nutrientes sobre la fisiología del cocolitofórido *Emiliania huxleyi*: proceso de calcificación y actividad enzimática.

IV. OBJECTIVES

1. To analyze the capability of phytoplankton to adapt to natural extreme environments by studying the different mechanisms and mutation rates that enable adaptation.

2. To study the genetic capacity of phytoplankton to adapt to different contaminants of anthropogenic origin: herbicides, antibiotics, heavy metals and different biocides. To difference the nature of the mutation that allows the resistance and the mutation rates conferring resistance.

3. To determine the differential capacity and maximal capacity of phytoplankton adaptation under diverse extreme conditions: temperature and different environmental pollutants.

4. To study the relative contribution of different evolutionary forces (genetic adaptation, chance and historical contingency) on the process of change in the cyanobacteria *Microcystis aeruginosa*, exposed to different environmental conditions.

5. To investigate the influence of ocean acidification and nutrient availability on the physiology of the coccolithophore *Emiliania huxleyi*: calcification and enzymatic activity.

V. MATERIAL Y MÉTODOS

"En el fondo, los científicos somos gente con suerte: podemos jugar a lo que queramos durante toda la vida"

(Lee Smolin. Físico teórico americano)

➢ ORGANISMOS EXPERIMENTALES

Entre los organismos experimentales se escogieron diferentes especies de organismos fitoplanctónicos agrupados según su nicho ecológico, y dentro de este, pertenecientes a distintos grupos taxonómicos.

I. FITOPLANCTON DE AGUA DULCE

Phylum Chlorophyta

Clase Trebouxiophyceae; Orden Chlorellales

Dictyosphaerium chlorelloides (Naum.) Komárek *et* Perman

Clase Chlorophyceae; Orden Sphaeropleales

Scenedesmus intermedius Chodat

Dictyosphaerium chlorelloides y *Scenedemus intermedius* son organismos eucariotas unicelulares pertenecientes al grupo de algas "verdes". Evolutivamente, las algas verdes son consideradas predecesoras de las plantas superiores terrestres por lo que han sido utilizadas en numerosos estudios fisiológicos y de toxicidad. Ambas especies contribuyen en gran medida a la producción primaria en aguas continentales por lo que han sido elegidas como representantes de este grupo.

Phylum Cyanobacteria

Clase Cyanophyceae; Orden Croococales

Microcystis aeruginosa (Kützing) Lemmerman

Las cianobacterias son organismos procariotas fotosintéticos que han habitado nuestro planeta durante los últimos 3.500 millones de años (Schopf y Packer 1987). Presentan una distribución global pudiendo ser encontradas en todo tipo de ambientes: agua dulce, salobres, marinas, así como ecosistemas terrestres. Determinadas condiciones ambientales como eutrofización o aumento de temperatura favorecen su crecimiento desencadenando una proliferación masiva o "bloom" (HABs: harmful algal blooms) dando lugar a modificación de la calidad del agua. Algunas de estas especies producen potentes toxinas capaces de originar efectos agudos y crónicos en el hombre, animales y vegetales (Codd *et al.* 1999a). Ambas características, unidas a la evidencia creciente sobre el aumento tanto espacial como temporal de la incidencia de blooms (Hudnell HK *et al.*), siendo más del 50% blooms tóxicos (Codd 1999b *et al.*) hacen que su estudio y control sea muy extendido. La especie seleccionada para esta tesis, *Microcystis aeruginosa* es considerada una de las especies más importantes por su distribución ubicua en todas las masas de agua y en embalses de abastecimiento de agua a las ciudades. Esta especie produce la toxina más abundante en las aguas continentales (Microcistina), que es un heptapéptido inhibidor de las enzimas Proteína Fosfatasas 1 y 2A. (Sivonen y Jones 1999).

II. FITOPLANCTON COSTERO:

Phylum Dinoflagellata

Clase Dinophyceae; Orden Prorocentrales

Prorocentrum triestinum Schiller

Prorocentrum triestinum es un dinoflagelado caracterizado por su capacidad para desarrollar crecimientos masivos en aguas tanto marinas como costeras, las denominadas "mareas rojas" (Toriumi 1980). Es capaz de producir toxinas, como el ácido okadaico y sus derivados, que liberan al medio dando lugar a importantes pérdidas tanto ecológicas como económicas en acuicultura.

Por ello, es importante el estudio de la evolución de esta especie ante determinadas condiciones de cambio ambiental.

Phylum Chlorophyta

Clase Prasinophyceae; Orden Chlorodendrales

Tetraselmis suecica (Kylin) Butcher

Tetraselmis suecica es un alga verde presente en ecosistemas marinos y costeros. Es capaz de crecer hasta concentraciones muy elevadas por lo que es usada como alimento en acuicultura fundamentalmente para la alimentación de rotíferos. Ha sido elegida como representante de las algas verdes en ecosistemas costeros.

Phylum Bacillariophyta

Clase Bacillariophyceae; Orden Naviculales

Phaeodactylum tricornutum Bohlin

Navícula *sp.*

Clase Bacillariophyceae; Orden Bacillariales

Nitzschia closterium (Ehrenberg) Smith

Phaeodactylum tricornutum, *Navicula sp.* y *Nitzschia closterium* son organismos eucariotas unicelulares pertenecientes al grupo de las diatomeas. Estos organismos presentan una cubierta de sílice llamado frústulo que presenta una diversidad de formas según especie. Son especialmente importantes en los océanos donde se calcula que proporcionan hasta un 45 % del total de la producción primaria oceánica (Mann 1999). En concreto, *Phaeodactylum*

tricornutum es frecuentemente usado en acuicultura debido a su capacidad nutritiva y capacidad de crecimiento. Asimismo, es capaz de dar lugar al desarrollo de "blooms" bajo distintas condiciones ambientales, contribuyendo en gran medida a la "bomba biológica" de captación de CO_2 atmosférico. Por ello, la respuesta de estas especies a distintos escenarios de cambio ambiental resulta interesante.

III. ALGAS SIMBIÓTICAS DE LOS CORALES

Phylum Dynoflagellata

Clase Dinophyceae; Orden Suessiales

Symbiodinium sp.

Los organismos de este género utilizados en esta tesis fueron aislados de corales. Estos organismos eucariotas unicelulares mantienen una relación simbiótica con los corales que habitan determinando el éxito de su supervivencia (Stanley 2006). Durante las últimas décadas distintos fenómenos de cambio climático y alteración de las condiciones ambientales, fundamentalmente temperatura, están dando lugar al denominado fenómeno de "blanqueamiento" de los corales (Sotka y Thaker 2005). Los corales pierden su coloración característica debido a la reducción en el número de simbiontes habitando en ellos, lo que corresponde a una pérdida de la relación simbiótica y con ello, afectación de la fisiología del coral así como una mayor predisposición a enfermedades. Por ello, la afectación de estos organismos ante distintos fenómenos de cambio climático es de gran interés. El género *symbiodinium* abarca un gran número de "clades" distintos presentando tolerancia diversa. Los dos organismos seleccionados en esta tesis pertenecen a los "clades" A2 y A3 (Stern *et al.* 2010).

IV. FITOPLANCTON OCEÁNICO

Las tres especies de fitoplancton marino utilizadas pertenecen al **Phylum Haptophycophyta**. Aunque la taxonomía de este phylum no llega a ser clara (Jordan *et al.* 2004), hemos seleccionado organismos pertenecientes a dos clases:

1. Clase Prymnesophyceae, Orden Isochrysidales, al que pertenecen las dos especies de cocolitofóridos:

   Emiliania huxleyi (Lohman) Hay *et* Mohler.

   Isochrysis galbana Parke

2. Clase Paulovophyceae, Orden Pavlovales, al que pertenece la tercera especie:

   Monochrysis lutheri (Droop) Green.

Los cocolitofóridos son organismos marinos unicelulares fotosintéticos capaces de producir placas de carbonato cálcico (principalmente calcita) denominadas "cocolitos" siendo considerados los mayores productores de calcita en el océano (Westbroek *et al.* 1989). Este grupo fitoplanctónico se caracteriza asimismo por su capacidad para la formación de blooms en aguas superficiales tanto costeras como oceánicas (Paasche 2002). Existen unas 280 especies de cocolitofóridos (Young *et al.* 2003) pero solo dos, entre ellas *Emiliania huxleyi* se conocen como formadoras de blooms en el presente periodo geológico (Jordan y Green 1994). *Emiliania huxleyi* se considera el cocolitofórido más abundante en los océanos actuales (Paasche 2002) distribuyéndose en la mayoría de los océanos exceptuando los océanos Ártico y Antártico (Paasche 2002; Tyrrel y Merico 2004) debido a su gran plasticidad genética (Iglesias-Rodríguez *et al.* 2002). Por ello, la biología de este cocolitofórido así como su papel en el ciclo del carbono es objeto de numerosos estudios y ha sido seleccionado para su estudio en esta tesis.

La especie *Isochrysis galbana* no da lugar a la formación de placas calcáreas. Se piensa que esta especie pudo perder de forma secundaria su habilidad para la formación de cocolitos (Billard e Inouye 2004). Tanto *Isochrysis galbana* como *Monochrysis lutheri* son ampliamente utilizados en la industria acuícola y fácilmente cultivables en laboratorio.

➢ CARACTERÍSTICAS COMUNES DE LOS ORGANISMOS SELECCIONADOS

Todos los organismos utilizados en esta tesis presentan reproducción asexual como única herramienta de reproducción. Por tanto, la mutación genética se considera su única fuente de variabilidad genética y la selección solo puede actuar sobre la variación que ha surgido "de novo" por mutación.

Para cada microorganismo utilizado los cultivos fueron previamente re-clonados a partir de una única célula antes del comienzo de los experimentos para evitar la inclusión de cualquier mutación espontánea previa que pudiera haber ocurrido en el cultivo. De esta manera se asegura asimismo la homogeneidad genética en el punto de partida.

➢ VENTAJA DEL USO DE MICROORGANISMOS EN EXPERIMENTOS DE EVOLUCIÓN (Elena y Lenski 2003)

1. Facilidad de propagar y enumerar.
2. Rapidez de reproducción lo que permite la realización de experimentos durante numerosas generaciones.
3. Permiten el manejo de poblaciones grandes en espacios pequeños facilitando el uso de réplicas.
4. Este tipo de organismos disponen de mecanismos que permiten mantenerse en estado de reposo (G_0), sin reproducirse, durante cierto tiempo. Por ello, los genotipos ancestrales pueden ser almacenados, mantenidos en reposo y salir de ese estado más tarde pudiendo ser comparados con los genotipos derivados.

5. Pueden elegirse organismos de reproducción asexual, por lo que se aumenta la precisión en los replicados.
6. La asexualidad permite asimismo realizar medidas de fitness debido a la asociación que puede mantenerse entre marcadores genéticos y un genoma concreto.
7. La manipulación de variables ambientales es fácil.
8. Existen abundantes bases de datos para multitud de especies que permiten realizar análisis genéticos precisos así como comparaciones posteriores.

➢ SISTEMAS EXPERIMENTALES: HERRAMIENTAS PARA EL ESTUDIO DE LA EVOLUCIÓN

I. ANALISIS DE FLUCTUACIÓN

"*During a pause in the music I found myself standing near a slot machine, watching a colleague putting dimes into it. Though losing most of the time he occasionally got a return. Not a gambler myself, I was teasing him about his inevitable losses, when he suddenly hit a jackpot... gave me a dirty look at walked away. Right then I began giving some thought to the actual numerology of slot machines; in doing so, it dawned on me that slot machines and bacterial mutations have something to teach each othe*r."

(Luria's autobiography: A Slot Machine, A Broken Test Tube).

Este párrafo define el "momento eureka" en que Salvador E. Luria (ganador, junto con Max Delbrück, del premio nobel en Fisiología y Medicina en 1969) descubrió la forma de demostrar experimentalmente la naturaleza de las mutaciones que permiten la adaptación de un organismo a un ambiente selectivo. Experimentos previos habían observado el resurgimiento de cultivos bacterianos al tiempo de ser expuestos a un agente bacteriófago. Este resurgimiento era asociado a la aparición de nuevas variantes resistentes a partir de genotipos sensibles y distintos autores planteaban opiniones

diversas sobre el momento de la aparición de la resistencia, antes o después de la exposición de la bacteria con el bacteriófago (Gratia 1921; D´Herelle 1926).

Sin embargo, el diseño experimental mediante el cual se podía determinar la forma en la que estas variantes aparecían, no se estableció hasta 1943, cuando Salvador Luria y Max Delbrück expusieron el magistral experimento del "Análisis de Fluctuación". Este experimento ha sido utilizado durante años para la determinación de la naturaleza de las mutaciones en distintos grupos de organismos desde bacterias hasta células animales (Coffino *et al.* 1975; Legarde AE 1989). Para la realización de este experimento, o al menos para la determinación de parámetros importantes como la tasa de mutación, es importante el uso de organismos unicelulares y asumir un 100% de eficiencia en cultivo en placa (Jones 1994a). Por ello, el experimento de Luria y Delbrück en cultivos de microalgas fue limitado durante muchos años a *Chlamydomonas sp* (Guilham y Levine 1962; Lee y Haughn 1980; Collard y Matagne 1990) puesto que esta microalga sí podía ser cultivada en placa de forma eficiente. Tratando de solucionar esta cuestión, López-Rodas *et al.* (2001), desarrollaron un diseño experimental sustituyendo el cultivo en placas de agar por medio líquido apropiado para el estudio de la mutación en microalgas incapaces de crecer en placa.

BASE TEÓRICA

El análisis de fluctuación es una combinación de procedimientos experimentales y estadísticos que permite determinar la naturaleza de la mutación que confiere la resistencia ante un determinado agente selectivo. Por ello, nos permite distinguir entre dos modelos:

- Modelo pre-selectivo: Con esta primera hipótesis se plantea que la resistencia ocurra por una mutación que aparece al azar, de forma espontánea antes de que el organismo entre en contacto con el agente selectivo. Por ello, la denominaron "mutation":

  "*There is a finite probability for any bacterium to mutate during its life time from "sensitive" to "resistant." Every offspring of such a mutant will be resistant, unless reverse mutation occurs. The term "resistant" means here that*

the bacterium will not be killed if exposed to virus, and the possibility of its interaction with virus is left open" (Luria y Delbrück 1943. p.193).

- Modelo post-selectivo: Con esta segunda hipótesis Luria y Delbrück plantearon la posibilidad de que la mutación que confiere la resistencia se produzca tras el contacto con el agente selectivo. Lo que ellos llamaron "adquired hereditary inmunity":
  "*There is a small finite probability for any bacterium to survive an attack by the virus. Survival of an infection confers immunity not only to the individual but also to its offspring. The probability of survival in the first instance does not run in clones. If we find that a bacterium survives an attack, we cannot from this information infer that close relatives of it, other than descendants, are likely to survive the attack*" (Luria y Delbrück 1943. p.193).

Luria y Delbrück plantearon estas dos hipótesis. En el caso de la resistencia debida a "mutación", la proporción de bacterias resistentes entre un número determinado de colonias bacterianas debería aumentar a lo largo del tiempo, debido a que los mutantes pueden aparecer en cualquier momento. Por ello, se encuentran grandes fluctuaciones que ayudan al estudio cuantitativo para la diferenciación de las hipótesis. Sin embargo, en el caso de la "inmunidad adquirida", la proporción de bacterias resistentes debe ser constante siempre que las condiciones de cultivo no cambien.

BASE METODOLÓGICA (adaptación a cultivos líquidos)

Para la distinción entre ambas hipótesis es necesario, por tanto, la determinación de la distribución de probabilidades de la aparición de bacterias resistentes en cada caso. Para ello, se establecen dos sets de experimentos (Figura 6). En el primer set, se cultiva un número elevado de cultivos, alrededor de 100 tubos (según el experimento), con un número bajo de células (10^2 células aproximadamente). El motivo de la baja densidad celular es el asegurar la ausencia de mutantes resistentes al principio del experimento. Asimismo es importante señalar que previamente al desarrollo del experimento las células deben ser re-clonadas a partir de una única célula para evitar incluir mutaciones

espontáneas acumuladas. Los cultivos crecen en condiciones no selectivas hasta alcanzar 10^5 células por cultivo. Durante este periodo de crecimiento algunas células podrían mutar y dar lugar a un genotipo resistente. Una vez que los cultivos alcanzan la concentración celular deseada de 10^5 células por cultivo se añade a todos los cultivos del set 1 el agente selectivo a la concentración letal previamente determinada. El agente selectivo será letal para aquellos genotipos sensibles pero las células resistentes podrán continuar su crecimiento. Simultáneamente, se prepara un segundo set (set 2) en el que un número menor de tubos (alrededor de 30) es inoculado directamente con 10^5 células procedentes de la misma población parental que aquellas del set 1. Este segundo set es inoculado directamente en presencia del agente selectivo a la misma concentración letal. Ambos sets se dejan crecer durante aproximadamente 60 días. Tras este tiempo, se determina el número de células resistentes en cada uno de los cultivos.

En el caso de que la resistencia al agente tóxico se desarrolle en respuesta directa a la presencia del agente, la probabilidad de cada bacteria de sobrevivir al bacteriófago es constante. Por ello, se esperará que la proporción de células resistentes se distribuya según una distribución de Poisson. En este caso, la varianza de las réplicas será igual al valor medio (V = M). En el caso de la aparición de la mutación por un fenómeno al azar, cada cultivo paralelo presenta una probabilidad distinta de desarrollar la resistencia en cada división celular. Por ello, habrá cultivos donde la resistencia se dé pronto en el tiempo y por tanto la mayoría de la progenie será resistente. En otros cultivos, la mutación que confiere la resistencia puede aparecer en una de las últimas divisiones celulares, resultando en un número bajo de células resistentes. Habrá cultivos en los que la mutación no tenga lugar, y por tanto todas las células fueron sensibles y no sobrevivieron a la concentración letal del agente selectivo. En el caso de la hipótesis de "mutación espontánea" la distribución de probabilidades, por tanto, no corresponde a una distribución de Poisson y consecuentemente la varianza de esos replicados será mayor que la media (V> M). El set 2 es considerado el control del experimento y mide la varianza de la población parental. La variación entre tubos muestra únicamente el error experimental. En el caso de este set, la probabilidad de que aparezcan mutaciones tanto pre-selectivas como post-selectivas al agente selectivo es alta, debido a que los cultivos fueron inoculados con una concentración celular elevada. Por ello, la

distribución de células resistentes correspondería nuevamente a una distribución de Poisson (V=M).

El ratio varianza/media del set 1 y 2, por tanto, es usado para distinguir entre la aparición de mutantes al azar (ratio varianza/media$_{set1}$> ratio varianza/media$_{set2}$) o mutación dirigida (ratio varianza/media$_{set1}$=ratio varianza/media$_{set2}$).

CÁLCULO DE LA TASA DE MUTACIÓN ESPONTÁNEA

El cálculo de la tasa de mutación espontánea es de considerable interés ya que ofrece elementos para comprender las capacidades evolutivas de los organismos (Klug y Cummings 1997). El análisis de fluctuación propuesto por Luria y Delbrück estableció una base para medir la frecuencia de los eventos mutacionales a partir de distintos estimadores (Luria y Delbrück 1943): el primer método hace uso del estimador P_0 y el segundo método hace uso del número medio de bacterias resistentes por cultivo. Desde la publicación de este artículo, trabajos posteriores discreparon en la mayor o menor fiabilidad del uso de los estimadores propuestos planteando nuevas aproximaciones como el uso de la mediana o el método de máxima verosimilitud (Lea y Coulson 1949; Li y Chu 1987; Stewart *et al.* 1990; Jones *et al.* 1994b; Stewart 1994; Asteris y Sarkar 1996). La elección de una u otra dependerá de la decisión del investigador considerando la que represente mejor la realidad de sus cultivos experimentales (Rosche y Foster 2000). Los trabajos presentados en esta tesis usan el estimador P_0 planteado por Luria y Delbrück (1943) como ya propuso López-Rodas *et al.* (2001) cuando se utilizaban cultivos en medio líquido. Este método se basa en el hecho de que el número de mutaciones en una serie de cultivos similares debe distribuirse según la distribución de Poisson. Por ello, el número medio de mutaciones por cultivo se calcula a partir de la proporción de cultivos del Set 1 que no contienen algas resistentes en el momento del test (P_0):

$$P_0 = e^{-\mu (Nt - N0)}$$

, donde N_t y N_0 corresponden al número de células final e inicial, respectivamente y μ corresponde a la tasa de mutación:

$$\mu = -\frac{Ln\, P_0}{N_t - N_0}$$

En estos trabajos se considera la tasa de mutación como el ratio de células mutantes por cada división celular (López-Rodas *et al.* 2001).

II. EXPERIMENTO DE RATCHET. DETERMINACIÓN DE LA MÁXIMA CAPACIDAD DE ADAPTACIÓN

BASE TEÓRICA

Reboud *et al.* (2007) plantearon un diseño experimental para el estudio del potencial de evolución de la resistencia de la microalga *Chlamydomonas reinhardtii* al herbicida simazina. Este protocolo permitía el estudio de la respuesta evolutiva de las poblaciones a presiones selectivas a largo plazo. Posteriormente, Orellana *et al.* (2008) modificaron este procedimiento para la obtención de microalgas presentando la máxima capacidad de adaptación ante distintos antibióticos, herbicidas y metales pesados. El diseño experimental utilizado en esta tesis parte de diseños previos, para estudiar la máxima capacidad de adaptación de las poblaciones ante dosis crecientes de un agente selectivo determinado así como el estudio de la capacidad de adaptación diferencial inter-específica.

Para la determinación de la máxima capacidad de adaptación de una población es importante tener en cuenta distintas cuestiones: a) la población inicial debe ser lo suficientemente elevada para asegurar una representativa variabilidad genética de la población evitando así el denominado "efecto fundador" (reducción del número de alelos de una población como consecuencia de una muestra inicial reducida); b) es necesario aplicar una presión de selección suficientemente fuerte como para asegurar el mantenimiento en la población de las mutaciones beneficiosas.

El protocolo del ratchet combina perfectamente ambas premisas. Asimismo, permite el mantenimiento de distintas dosis selectivas. De este modo, se asegura el mantenimiento del crecimiento a dosis inferiores durante el cual es posible que pueda surgir y propagarse una mutación beneficiosa. Estas características confieren al sistema de Ratchet una capacidad importante para el estudio de la respuesta evolutiva de un organismo ante una presión selectiva determinada.

BASE METODOLÓGICA

El protocolo de Ratchet se basa en el mantenimiento de un equilibrio entre la presencia de poblaciones elevadas y una fuerte presión selectiva, la cual es mantenida gracias a la transferencia de las poblaciones resistentes a dosis crecientes del agente selectivo. Cada una de las transferencias realizadas corresponde a un ciclo de Ratchet.

El diseño experimental consta de tres dosis selectivas crecientes y una población control mantenidos en cada ciclo de Ratchet (Fig. 7). Las dosis iniciales de las que se parte son las mismas para todas las especies, puesto que quieren ser comparadas, y su determinación se realiza a través de experimentos previos de dosis-efecto. Cada uno de los tratamientos se cultiva por triplicado considerando cada replica como una población independiente. Todos los experimentos se fundan a partir de cultivos en fase exponencial de crecimiento. Cada replicado es inoculado con una concentración celular elevada (≈ 300.000 células ml^{-1} según la especie) y es mantenido bajo condiciones selectivas durante 20 días. Tras ese tiempo, se determina la concentración celular en cada replicado y se compara con el crecimiento en las poblaciones control. Para considerar el paso al siguiente ciclo de Ratchet, a la siguiente concentración selectiva, la población experimental debe haber alcanzado una concentración celular al menos igual que la de la población control. En el caso de que la densidad celular alcanzada no sea la adecuada, los cultivos se mantendrán a la misma concentración durante otro ciclo de ratchet, en espera de la siguiente determinación. Es importante señalar que cada replicado se considera una población independiente. De esta manera, la transferencia de cada uno de ellos al nivel de selección siguiente será independiente del resto de replicados. 20 días se consideran tiempo suficiente para que los cultivos alcancen una concentración celular elevada. Obviamente, el tiempo empleado por cada especie para

alcanzar la misma densidad celular es diferente, puesto que sus tasas de crecimiento son distintas. Tras este periodo las poblaciones control pueden encontrarse en la fase estacionaria en el momento de realizar el contaje celular, en el caso de las poblaciones con mayor tasa de crecimiento. Pero de cualquier manera, este tiempo es necesario puesto que debido a la reducción de la tasa de crecimiento en las poblaciones sujetas a una fuerte presión de selección, el tiempo necesario para alcanzar la misma concentración celular es elevado. El final del protocolo de selección se considera tras 6 ciclos de ratchet sin observar crecimiento de los cultivos experimentales. El máximo nivel de selección que permite el crecimiento de esa cepa determinada se considera la máxima capacidad de adaptación a un agente selectivo concreto. Dependiendo del agente selectivo y de la capacidad de cada especie para la evolución de resistencia genética, el número de ciclos de ratchet será distinto.

Mediante las ecuaciones de Novick y Szilard (1950), somos capaces de determinar el número de generaciones acontecidas durante los experimentos de Ratchet lo que nos permite comparar entre distintos replicados.

$$N_t = N_0\, 2^{t/T}$$

$$g = t / T$$

donde N_t = nºde células a tiempo t, N_0 = nº de células en el momento inicial, t = tiempo, T = tiempo de generación y g = nº de generaciones.

Este diseño experimental permite asimismo elucidar si la adaptación se produjo por medio de procedimientos genéticos o de lo contrario, derivó como resultado de una adaptación fisiológica. Para ello, nos fijaremos en la evolución diferencial de cada uno de los replicados a cada dosis concreta. Puesto que cada réplica es considerada como una población independiente la dinámica de resistencia en cada una puede ser distinta: a) en el caso de que la resistencia al agente selectivo ocurra por medio de aclimatación fisiológica, todos los replicados presentarán la misma probabilidad de presentar la resistencia. Por ello, el número de generaciones requeridas para alcanzar el crecimiento

deseado será el mismo en todos los replicados; b) si por el contrario, la aparición de la resistencia aparece debido a mecanismos genéticos, el número de generaciones necesarias para alcanzar la resistencia variará entre réplicas. Obviamente, es posible que ambos fenómenos acontezcan al mismo tiempo. En los primeros ciclos de selección (niveles bajos de presión selectiva), las mutaciones que confieren débil resistencia puedan presentarse a una frecuencia más elevada. Por tanto, todos los replicados podrían mostrar crecimiento a un tiempo determinado similar sin ser debido únicamente a un fenómeno de aclimatación fisiológica. Sin embargo, en ciclos posteriores donde la presión selectiva es mayor se requiere mecanismos de resistencia más complicados para asegurar la supervivencia, que no pueden ser asegurados por mera adaptación fisiológica. Es posible que estos mecanismos no se produzcan en todas las poblaciones, o si se producen, sea en tiempos diferentes. Por ello, se observara diversidad en la evolución de los distintos replicados.

El protocolo de Ratchet permite por tanto explorar las distintas posibilidades del fitoplancton para adaptarse genéticamente a distintos agentes selectivos así como determinar la capacidad diferencial de adaptación entre los distintos grupos fitoplanctónicos.

III. CONTRIBUCIÓN DE LAS FUERZAS DE EVOLUCIÓN

“ You press the rewind button and, making sure you thoroughly erase everything that actually happened, go back to any time and place in the past (...). Then, left the tape run again and see if the repetition looks at all like the original. If each replay strongly resembles life´s actual pathway, then we must conclude that what really happened pretty much had to occur. But suppose that the experimental versions all yield sensible results strikingly different from the actual history of life (...)”.

(Stephen Jay Gould, Wonderful life 1989)

BASE TEÓRICA

Con esta idea, Stephen Jay Gould, defensor de la importancia de la contingencia histórica (Gould y Lewontin 1979), imaginó perfectamente como podría ser el experimento perfecto para demostrar la contribución de ésta en el cambio evolutivo. Gould dedicó parte de su investigación al estudió de uno de los yacimientos fósiles más importantes a nivel mundial: Burguess Shale. Observó que la extraordinaria diversidad observada al inicio del periodo Cámbrico no se mantuvo tras la subsiguiente extinción masiva que aconteció y sólo algunas de esas especies sobrevivieron y evolucionaron. Con esta apreciación, Gould se planteaba la repetitividad de la evolución. Si se volviera al punto de inicio, hace 488 millones de años, con las mismas especies de partida, ¿se observaría el mismo resultado?, o incluso, ¿podríamos estar aquí planteándonos esa pregunta si por algún motivo, los primeros cordados no hubieran sobrevivido? Si el cambio evolutivo dependiera única y exclusivamente del poder de la selección natural, nos encontraríamos siempre ante la misma situación tras correr la cinta de nuevo. Sin embargo, si el resultado no fuera tan selectivo, si tanto las formas adaptadas como las no adaptadas presentaran la misma probabilidad de evolucionar, el resultado no podría ser predecido.

De esta manera, Gould defendía el efecto de la historia, la cual, podría constreñir o promover resultados evolutivos determinados según la genética y desarrollo evolutivo del genotipo concreto.

Basándose en el experimento teórico planteado por Gould (1989), Travisano *et al.* (1995a) plantearon un experimento robusto para la evaluación y cuantificación de la contribución de tres componentes principales del cambio evolutivo: adaptación, azar e historia, sobre la evolución de distintos caracteres bajo una presión selectiva a largo plazo. Obviamente, un experimento tal cual como el planteado por Gould es imposible. Pero Travisano *et al.* (1995a) plantearon un experimento por el cual se conseguía el mismo objetivo. Consistía en la propagación simultánea de réplicas de aislados independientes en ambientes idénticos y la comparación de un carácter concreto entre las poblaciones ancestrales y derivadas. De este modo, las diferencias entre valores

medios iniciales y derivados se explicarían como resultado del efecto de las distintas fuerzas evolutivas (Fig.8). En el punto inicial, se espera que la diferencia de la media entre réplicas sea idéntica dentro de los límites estadísticos relativos al error experimental. Tras la fase de propagación, podría ocurrir que no se observen diferencias entre la población ancestral y derivada. Con esto se concluiría que no ha tenido lugar cambio evolutivo alguno (Fig.8.1a). Si no observamos cambio significativo en la media del carácter pero se observa un aumento significativo de la varianza nos encontramos ante la influencia única del azar, bien sea por deriva genética o fenómenos de mutación (Fig.8.1b). Podría ocurrir que se observara una variación en el valor medio pero sin diferencias significativas entre aislados. En este caso nos encontraríamos ante la contribución única de la adaptación. Sin embargo, podría ser tanto que ese carácter determinado haya sido diana de la selección natural o esta correlacionado con algún carácter que ha sido seleccionado (Fig.8.1c). Otra opción sería un cambio tanto en el valor medio como en la varianza. En este caso, nos encontraríamos ante la contribución combinada de la adaptación y azar (Fig.8.1d).

Para poder considerar el efecto de la contingencia histórica es importante el uso de genotipos distintos para poder comparar la evolución paralela de cada uno de ellos en ambientes idénticos. Puede ocurrir que la variación inicial en un determinado carácter entre los distintos genotipos sea eliminada en la población derivada. Todos ellos convergen en un mismo resultado evolutivo, no pudiendo reconstruir el genotipo ancestral a través del carácter determinado. De esta manera significaría que la huella histórica ha sido borrada debido al efecto de la adaptación, azar, o ambos (Fig. 8. 2a). Si por el contrario, las diferencias entre las poblaciones iniciales y derivadas se mantienen al final del tiempo de propagación, significa que la huella de la historia es mantenida (Fig. 8.2b).

Es importante señalar que la contribución de las distintas fuerzas evolutivas no es exclusiva por lo que podrían participar todas al mismo tiempo en el proceso evolutivo de un carácter determinado.

BASE EXPERIMENTAL

El diseño experimental planteado en esta tesis se basa en el modelo presentado por Flores-Moya *et al.* 2008, el cual a su vez se basa en aquél presentado por Travisano *et al.* (1995a).

Aunque ya se ha señalado anteriormente como punto común en los experimentos de evolución, cada una de las cepas es re-clonada a partir de una única célula para asegurar la homogeneidad genética en el punto de partida. De cada una de las cepas se cultiva un número determinado de réplicas (15 en nuestro caso concreto) en las condiciones iniciales y se determina la media de un carácter concreto (esa medida corresponde al valor de la población ancestral). En ese momento, cada una de las réplicas se transfiere a dos cultivos independientes para conseguir dos poblaciones idénticas. Una de ellas será considerada como la población experimental, la cual será transferida a las nuevas condiciones de cultivo selectivas y propagada durante un número determinado de generaciones (ver figura 9). La otra será mantenida en las condiciones iniciales actuando como población control. Cada una de las réplicas debe ser transferida a medio fresco cada 21 días aproximadamente para la renovación de nutrientes.

La determinación de los caracteres de interés se realiza cada 4 meses y los valores obtenidos tras 12 meses de propagación son comparados con los valores iniciales para la determinación de la contribución de las distintas fuerzas evolutivas. La determinación cuantitativa de la contribución de la adaptación es definida a través de cambios en el valor medio de los valores iniciales y finales de un determinado carácter. Para ello se pueden utilizar distintas aproximaciones estadísticas de comparaciones de medias como es la distribución t de Student o su alternativa no paramétrica, el test de la U de Mann Whitney. El efecto del azar e historia se pueden estimar a través de un ANOVA anidado mediante el cual se determina la varianza asociada a cada factor estudiado en concreto. En nuestro caso, contamos con dos valores de variación (cepa y réplicas, factor anidado en el anterior). De tal manera, la contribución del componente

azar al proceso evolutivo corresponde a la varianza medida entre réplicas. Por otro lado, la varianza entre cepas permite estimar la contribución de la historia en el proceso evolutivo. Para la cuantificación de la contribución del azar y la historia se utiliza la raíz cuadrada de la media cuadrática de cada uno de los componentes de la varianza. El uso de la raíz cuadrada se explica para poder usar unidades comparables con la cuantificación de la adaptación. Asimismo se calculó el intervalo de confianza al 95% para la varianza. Según el diseño experimental planteado en nuestro experimento, la adaptación inicial debe ser cero y el azar inicial corresponderá al error de muestreo, puesto que los replicados no han tenido tiempo de adaptarse o diferir. Las diferencias iniciales entre genotipos reflejan el efecto de la historia inicial debido a que corresponden a distinta filogenia.

Determinación del cambio fisiológico o genético

El diseño experimental presentado permite asimismo la diferenciación de la naturaleza del cambio, en el caso de que tenga lugar. Si el proceso de adaptación se produjo por como resultado de la selección de nuevas variantes genéticas o por el contrario, fue debido a un proceso de aclimatación. Para ello, la población experimental es transferida de nuevo a las condiciones iniciales y mantenida en éstas durante un mes. De la misma manera la población control se mantuvo en las mismas condiciones durante un mes más (13 meses en total). Si el valor medio del carácter concreto coincide con aquél del control significará que el proceso de cambio es debido a un fenómeno de aclimatación puesto que la población puede volver de forma rápida al valor inicial. Sin embargo, si el proceso de adaptación se produjo mediante la selección de las variantes adaptadas, la población adaptada no podrá volver a presentar los valores iniciales tras un mes de cultivo en las condiciones de partida y por tanto se puede concluir que ha tenido lugar un cambio genético.

CONSIDERACIONES DEL DISEÑO EXPERIMENTAL

Dentro del concepto de "Contingencia histórica" Gould (1989) formuló dos nociones distintas, aunque ambas pueden ser vistas complementariamente (Beatty 2006):

- La "imprevisibilidad" de la contingencia: partiendo de los mismos orígenes idénticos los resultados son impredecibles:

  "Any reply of the tape would lead evolution down a pathway radically different from the road actually taken (...)". (p.51).

- La "Dependencia-causal": el resultado final dependerá de los estados previos de los que preceda. Es por ello dependiente o contingente de cualquier cambio previo. La imborrable huella de la historia:

  "A *historical explanation rests on an unpredictable sequence of antecedent states where any major change in any step of the sequence would have altered the final result".* (p.283).

El diseño experimental planteado por Travisano *et al.* (1995a) y el usado en esta tesis alude únicamente a la noción de "dependencia causal". Se apela a la historia desde el punto de vista de distintos resultados evolutivos a partir de distintos puntos ancestrales. Hacemos lo mismo que en la película de Frank Capra "It´s a Wonderful life" y en la que Gould se inspira. Travisano *et al.* (1995b) plantearon un trabajo simultáneo en el que sí distinguían la opción de "imprevisibilidad" mediante el estudio de las diferencias genotípicas en 12 aislados que partiendo del mismo ancestro común seguían convergiendo en la fitness tras 1000 generaciones. Sin embargo, la fitness era distinta al cultivarlo en medio distinto del que partieron. Estas diferencias pueden ser debidas al orden en el que las mutaciones aparecen en la historia evolutiva de cada población, aunque estas lleven al mismo resultado. Como plantearon Gould y Lewontin (1979) (p.593): " *Subpopulations within a species often develop different adaptations as solutions to the same problem*". En el mismo sentido Lenski *et al.* (1991) afirmaron (p.1337): *"It is possible that the populations have evolved to similar mean fitnesses, but by different physiological adaptations," y "it may be*

possible to distinguish the populations from each other on the basis of differences under other environmental conditions."

Otra diferencia importante entre el experimento de Gould y el aquí planteado es que no se distingue entre azar e historia en el mismo sentido en que Gould lo hizo. Gould distinguió entre historia y "efectos estocásticos". Para él, los efectos estocásticos eran los efectos derivados de la deriva genética y error de muestreo. Sin embargo, consideraba que el fenómeno de mutación al azar y orden mutacional son fuentes de contingencia histórica puesto que el resultado final dependerá de éstos. Sin embargo, el experimento diseñado por Travisano *et al.* (1995a) y el usado en esta tesis incluyen en la misma categoría "azar" tanto el error debido al muestreo experimental como mutación al azar y orden mutacional. Por tanto, "azar" se superpone con contingencia histórica.

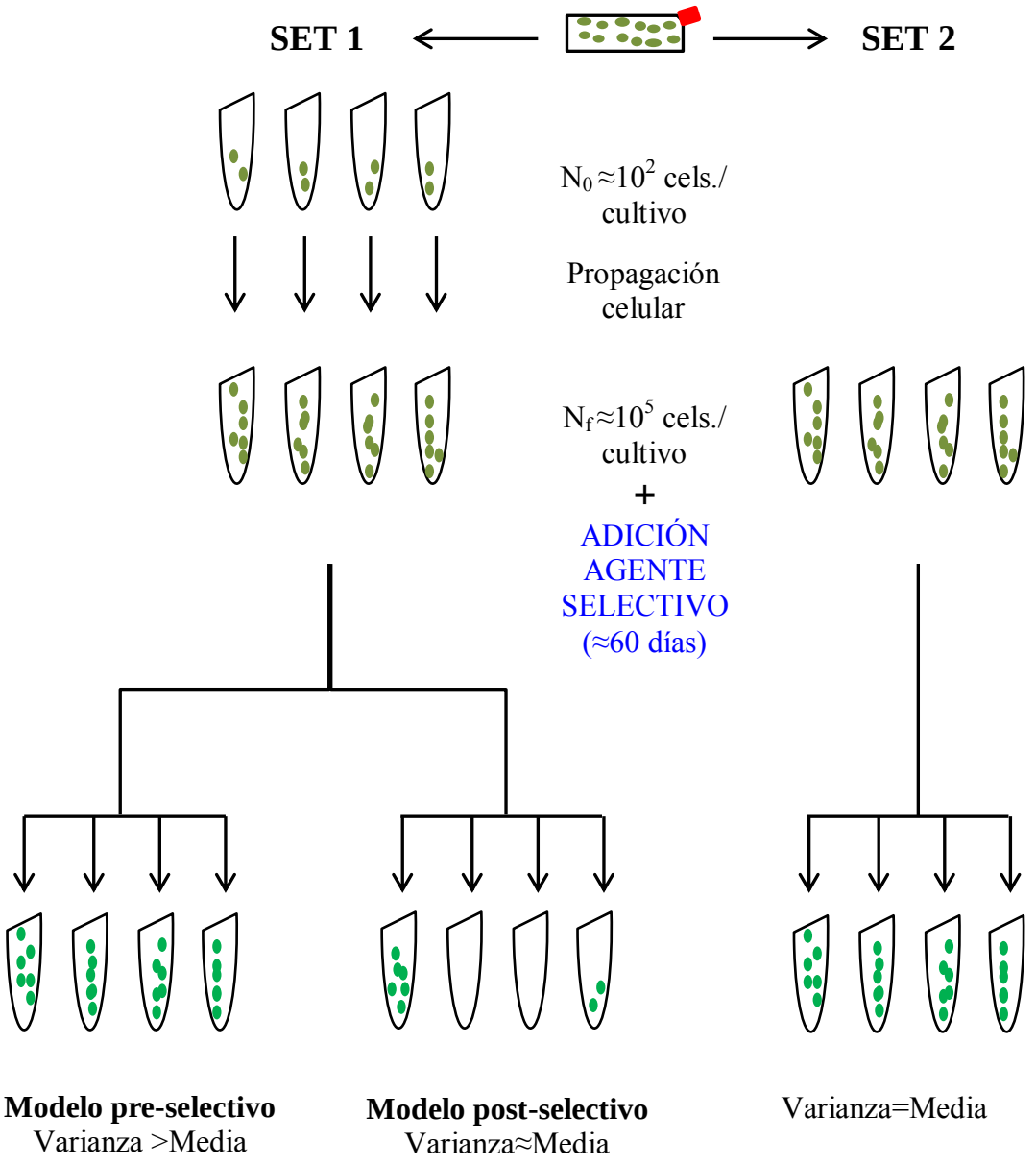


Figura 6. Esquema del análisis de fluctuación de Luria y Delbrück adaptado para cultivo en medio líquido (López-Rodas 2001). Los puntos verdes oscuros y claros representan las células con genotipo sensible y resistente respectivamente. En el set 1 se cultivan 10^2 células por cultivo en medio no selectivo y se dejan crecer hasta alcanzar 10^5 células. En ese momento se añade el medio selectivo y simultáneamente se funda el set 2, con 10^5 células procedentes de la misma población parental. Si las células resistentes aparecen en respuesta directa al agente selectivo, el número de células por cultivo será similar y V≈M. Si las células resistentes aparecen de forma espontánea antes de entrar en contacto con el agente selectivo se observará una importante fluctuación en el número de células entre cultivos y V>M. El set 2 es el control experimental (V≈M).

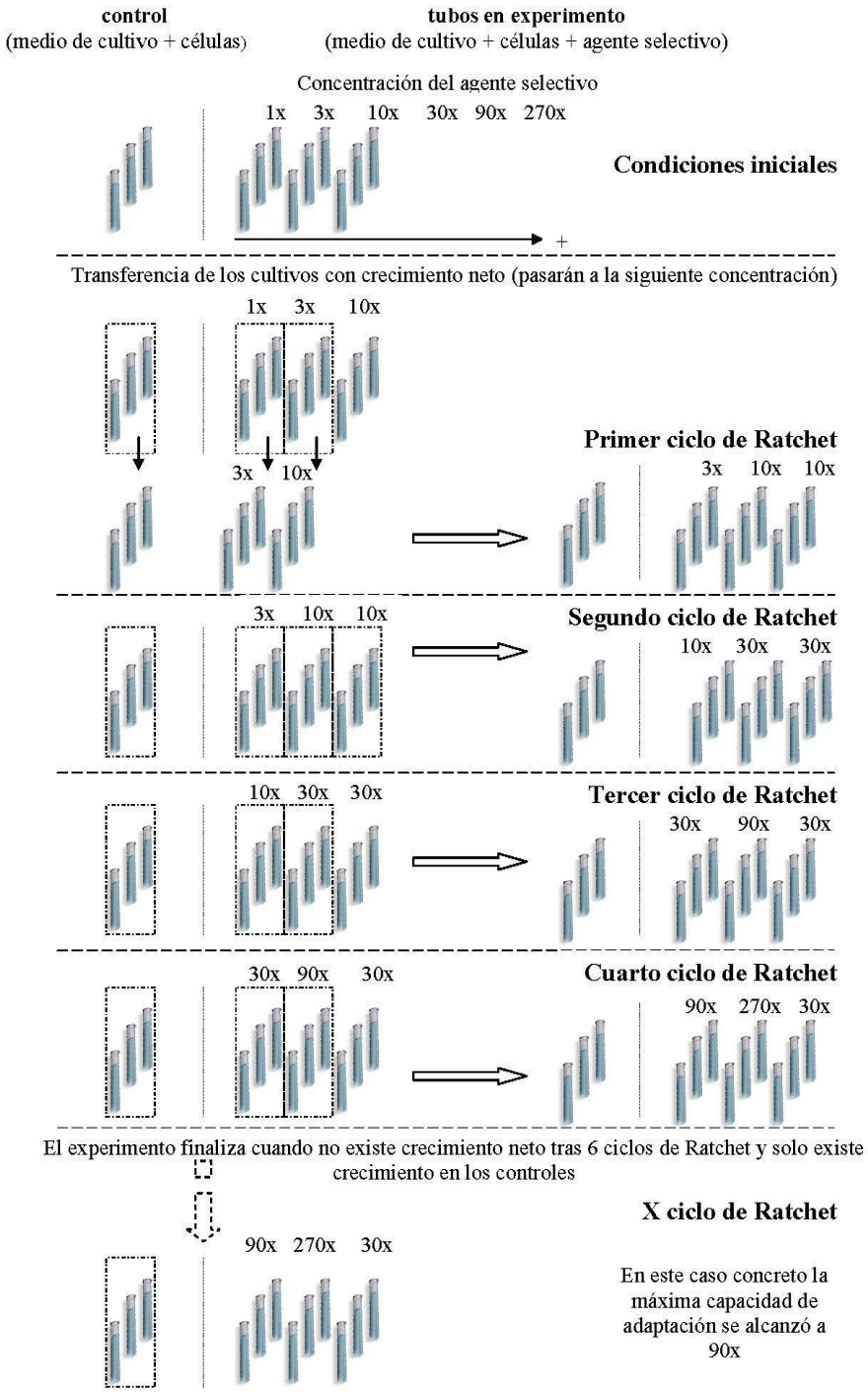


Figura 7. Esquema del experimento de Ratchet. Cada ciclo de ratchet consta de tres réplicas del control y tres réplicas de cada condición experimental a las dosis propuestas. La transferencia a la siguiente dosis se realiza cuando el tubo experimental alcanza el mismo crecimiento que los controles. Los tubos que no alcanzan el crecimiento deseado se mantienen en las mismas condiciones hasta el siguiente ciclo. La máxima capacidad de adaptación se considera a la dosis antes de la cual no se aprecia crecimiento tras 6 ciclos de ratchet.

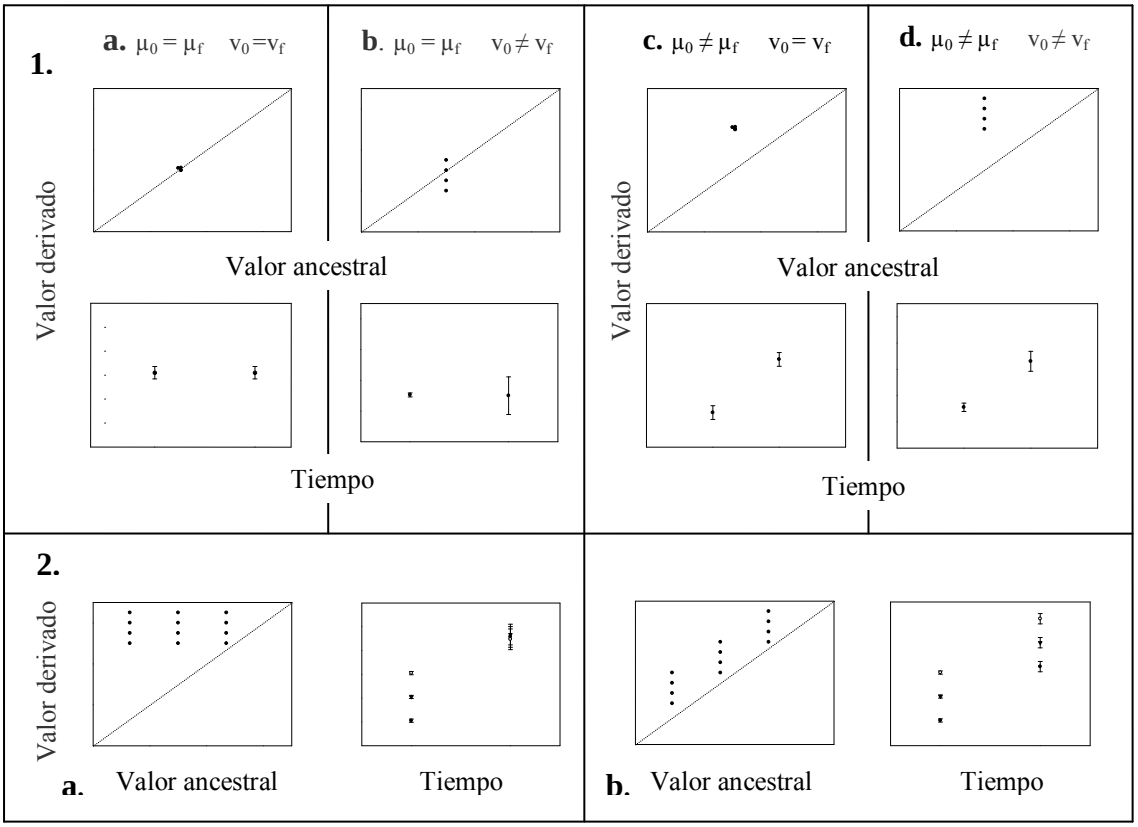


Fig.8. Representación esquemática de los efectos debidos a adaptación, azar e historia en el cambio evolutivo. 1. a) No existe cambio evolutivo; b) Efecto debido al azar; c) Efecto debido únicamente a la adaptación; d)Efecto debido a la influencia del azar y la adaptación. 2. Análisis de la contribución de la historia a) El efecto inicial de la historia es eliminado el afecto de la adaptación y el azar b) El efecto inicial de la historia se mantiene así como la participación de la adaptación y azar.

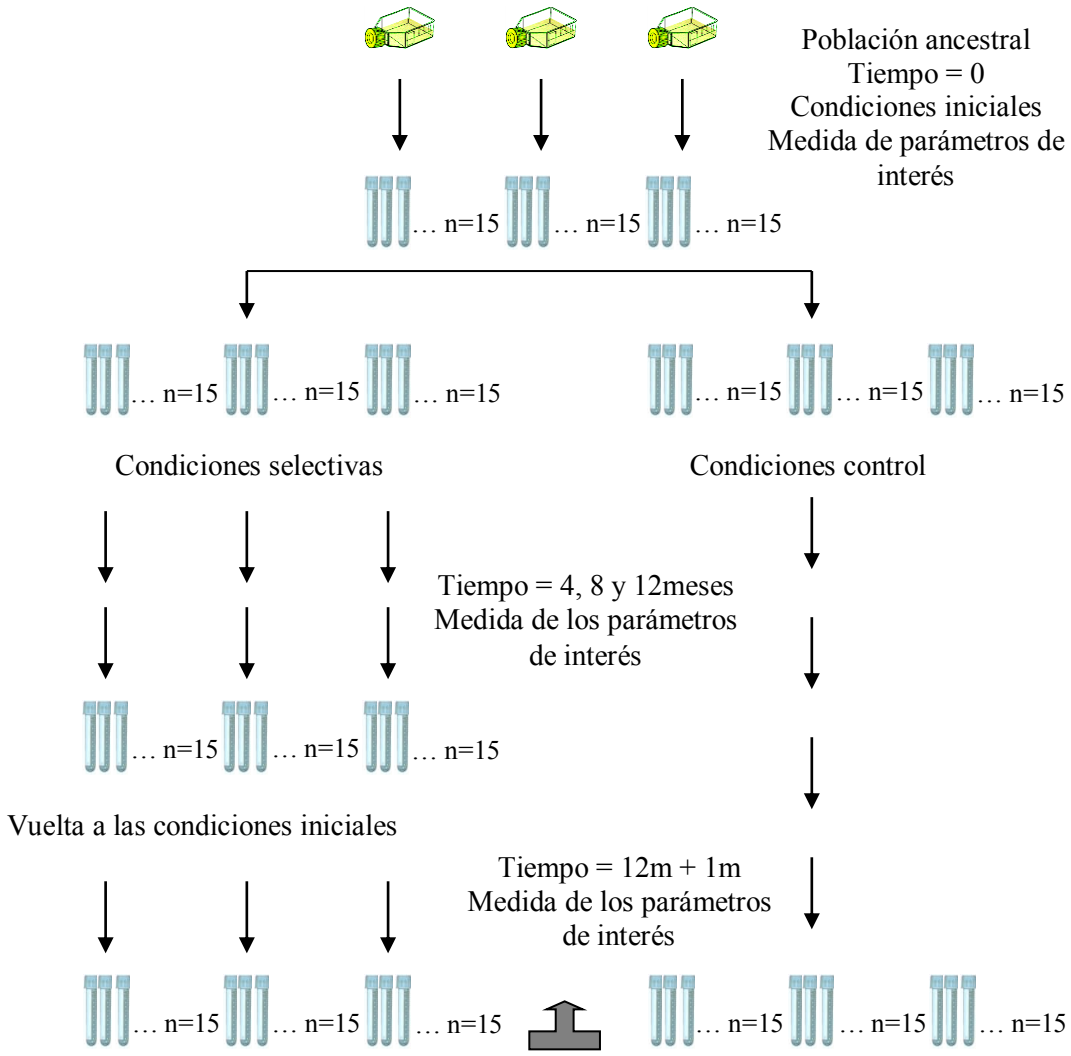


Fig. 9. Representación esquemática del diseño experimental. Se fundan 15 aislados para las condiciones control y 15 aislados para las condiciones selectivas. Cada cultivo es mantenido mediante transferencias seriadas en medio fresco durante 12 meses (medio selectivo o medio no selectivo, según se trate del experimento o población control respectivamente). Los parámetros de interés son determinados al principio del experimento, 4 meses, 8 meses y a los 12 meses. Para discriminar entre cambio genético o aclimatación los cultivos son transferidos de nuevo a las condiciones iniciales y mantenidos durante un mes. Los parámetros de interés son determinados nuevamente.

- **SISTEMA CARBONATO EN EL OCÉANO Y HERRAMIENTAS PARA LA MANIPULACIÓN DE LA QUÍMICA DEL CARBONO EN EL LABORATORIO**

SISTEMA CARBONATO EN EL OCÉANO

Aunque la composición de agua de mar varía de un océano a otro, presentando variaciones en densidad y otros parámetros físicos y químicos, su composición relativa está bien definida (Millero *et al.* 2008). De entre todo el conjunto de sales disueltas en el agua de mar, siendo NaCl la más predominante, tan solo el 0.7 % corresponde al conjunto de especies ácido-base que determinarán cambios en la acidez del agua siendo el ácido carbónico y el ácido bórico las especies predominantes (Dickson 2010). Para entender el equilibrio ácido-base del agua de mar es fundamental conocer el sistema carbonato (Schulz *et al.* 2009).

PARÁMETROS ANALÍTICOS PARA EL SISTEMA CARBONATO EN AGUA DE MAR

La concentración individual de las distintas especies del sistema ácido-base en agua de mar no pueden ser medidas directamente. Normalmete la concentración es inferida a partir de la combinación de **a**. medidas analíticas realizadas en la muestra de estudio y **b.** constantes de disociación empíricas altamente dependientes de la temperatura y salinidad (Dore *et al.* 2009; Dickson 2010). Los cuatro parámetros del sistema carbonato que pueden ser determinados experimentalmente en nuestra muestra de agua de mar son:

- $\boldsymbol{pCO_2}$
- **DIC**
- $\boldsymbol{A_T}$ **o TA**
- **pH**

pCO_2

La presión parcial de dióxido de carbono en aire en equilibrio con el agua de mar a una determinada temperatura es una medida del grado de saturación de la muestra de agua con CO_2 gas. Es una función fuertemente dependiente de la temperatura (Dickson 2010).

DIC (Carbono Inorgánico Disuelto Total)

Suma de todas las especies de carbono disueltas en agua de mar. En el océano, el dióxido de carbono disuelto en agua de mar existe principalmente en tres formas orgánicas agrupadas en su conjunto bajo la denominación de DIC o C_T (Carbono Inorgánico Disuelto Total): dióxido de carbono disuelto (CO_2), el cual incluye también el ácido carbónico (H^-CO_3), puesto que ambas formas son interconvertibles y representan alrededor del 1% del total; ión bicarbonato (HCO_3^-) representando el 91% y el ión carbonato (CO_3^-) representando el 8% restante (Raven *et al.* 2005; Hurd *et al.* 2009; Dickson 2010). Suele ser expresado en moles por Kg de solución.

$$DIC = [H_2CO_3^*] + [HCO_3^{2-}] + [CO_3^{2-}]$$

Alkalinidad Total (T_A)

Es la concentración total de bases débiles en el agua capaces de neutralizar la concentración total de iones hidrógeno $[H^+]$ (Hurd *et al.* 2009; Schulz *et al.* 2009). Suele ser expresado en moles por Kg de solución.

$$T_A = [HCO_3^-] + 2[CO_3^{2-}] + [OH] - [H^+] + (\ldots)$$

(…) representa las especies ácido-base secundarias: $[B(OH_4^-)] + [HPO_4^{2-}] + 2[PO_4^{3-}] + [H_3SiO_4] + [NH_3] + [HS^-] - [H_3PO_4] - [HSO_4^-] - [HF]$

Estos dos últimos parámetros tienen la ventaja de ser independientes de la temperatura y presión por lo que son conservados durante los distintos fenómenos de mezcla de masas de agua (Hurd *et al.* 2009).

Concentración de Iones Hidrógeno [H^+]

La concentración de iones hidrógeno en una muestra se define como pH:

$$pH = - \log [H^+]$$

Es muy importante considerar que el pH de una muestra depende altamente de la presión y temperatura (Dickson 2010; Schulz *et al.* 2009). En la actualidad el pH medio global en la superficie oceánica oscila alrededor de 8.2. Sin embargo puede presentar oscilaciones de ±0.3 unidades asociados a factores locales, regionales y climatológicos. Los dos factores principales que determinan la distribución espacial de pH son la temperatura superficial y el "*upwelling*" de aguas profundas ricas en CO_2. Zonas de temperaturas bajas (que aumentan la solubilidad del gas) y zonas en las que tienen lugar fenómenos de "*upwelling*" se caracterizan por presentar pH reducidos (Raven *et al.* 2005).

Si dos de estos parámetros son conocidos se puede calcular el resto de los parámetros del sistema carbonato teniendo en cuenta la concentración de nutrientes, temperatura, salinidad y presión (Dore *et al.* 2009). DIC and A_T son los parámetros más recomendados debido a que ambos pueden ser medidos con precisión y exactitud y sus concentraciones no cambian con la temperatura y presión (Dickson 2010).

Otro concepto importante en el contexto de acidificación oceánica y que afecta fundamentalmente a los organismos calcificadores es el **Estado de Saturación del carbonato cálcico (Ω)**. Los organismos calcificadores son capaces de precipitar carbonato cálcico ($CaCO_3$) para la formación de estructuras sólidas a partir del ión Ca^{2+} y CO_3^{2-}. Pero al igual que son formadas son vulnerables a disolución al menos que las aguas contengan concentraciones saturantes de carbonatos.

Estado de Saturación del carbonato cálcico (Ω).

El estado de saturación del carbonato cálcico se define como:

$$\Omega = ([Ca^{2+}]_{SW}[CO_3^{-}]_{SW})/K_{sp}^{*}$$ (Schulz *et al.* 2009),

donde $[Ca^{2+}]_{SW}$ y $[CO_3^{-}]_{SW}$ corresponden a las concentración de ión calcio e ión carbonato presente en agua a la temperatura, presión y salinidad *in situ*. K_{sp}^{*} representa la constante de solubilidad del carbonato cálcico. Cuando Ω es 1, el mineral se encuentra en equilibrio, no se está formando ni disolviendo. A la profundidad a la que esto ocurre, se le denomina *horizonte de saturación o lisoclina*. Por encima del *horizonte de saturación* Ω presenta un valor mayor que 1 y $CaCO_3$ no se disuelve fácilmente. Por debajo del *horizonte de saturación* Ω presenta un valor menor que 1 y $CaCO_3$ se disolverá (Raven *et al.* 2005). Las dos formas principales en las que se presenta el carbonato cálcico son la aragonita y la calcita las cuales presentan distintas constantes de solubilidad y por tanto distintos estados de saturación Ω_{calc} and Ω_{arg} respectivamente. La aragonita es 1.5 veces más soluble que la calcita (Dickson 2010) presentando su *horizonte de saturación* más próximo a la superficie. En ocasiones, la calcita puede presentar una forma más soluble debido a la sustitución de calcio por magnesio.

HERRAMIENTAS PARA LA MODIFICACIÓN DEL EQUILIBRIO DEL SISTEMA DEL CARBONO EN AGUA DE MAR

Para estudiar los efectos del aumento de CO_2 atmosférico y la acidificación oceánica en la fisiología del fitoplancton se requiere un control cuidadoso de todos los parámetros del sistema carbonato. Antes de desarrollar el diseño experimental de perturbación de CO_2 es importante tener en cuenta distintos aspectos:

- La interdependencia del sistema carbonato: La variación de uno de los componentes del sistema conduce a modificaciones de uno o más componentes del mismo (Gattuso y Lavigne 2009) siendo imposible variar un parámetro y mantener constante el resto de ellos.

- Impacto de procesos biológicos o físicos: El ajuste de la química del carbono se lleva a cabo al inicio del experimento. Todos aquellas alteraciones derivadas de procesos biológicos (ej. fotosíntesis, respiración y calcificación), así como procesos físicos (ej. cambios de temperatura o intercambio gaseoso con el medio externo) durante el desarrollo del experimento, no pueden ser controladas (Gattuso y Lavigne 2009, Hurd *et al.* 2009). Por ello, es aconsejable la realización de estos experimentos con densidad celular baja para evitar alteraciones grandes del sistema en este sentido.

Para controlar el primer punto, es importante la elección del método más adecuado de manipulación del sistema carbonato Los distintos métodos modifican la especiación de los iones carbonato de forma diferente pudiendo influenciar la respuesta biológica del fitoplancton (Fig. 10). Existe un gran rango de respuestas en especies concretas durante los experimentos de manipulación de pH (Hurd *et al.* 2009). Entre los posibles factores causantes de esta variabilidad (diferencias en fisiología, periodo de aclimatación, duración del estudio, época del año) se considera que el método de manipulación del pH elegido puede afectar debido a la distinta afectación del sistema carbonato.

Por ello, la elección de la mejor técnica de manipulación será muy importante a la hora de la interpretación de nuestros resultados. Asimismo, la elección de cada sistema dependerá del objetivo del experimento y duración del mismo, las variables de interés que quieren ser analizadas, la especie en estudio así como los volúmenes de los cultivos utilizados (Shi y Morel 2009; Hurd *et al.* 2009; Gattuso y Lavigne 2009).

Los métodos de manipulación pueden agruparse entre (véase Gattusso *et al.* 2010 para más detalle):

- Métodos que modifican DIC manteniendo A_T constante. En este grupo se incluye: burbujeo o inyección de distintos gases (CO_2 puro o CO_2 combinado con aire atmosférico o con otros gases); adición de agua de mar con una concentración alta de CO_2 (la concentración deseada se alcanzará por la mezcla de las dos masas de agua); adición de CO_3^{2-} o HCO_3^- (que provoca el aumento

de DIC) seguido de la adición de un ácido fuerte, generalmente HCl (que cancela el aumento de A_T producido tras la adicción de CO_3^{2-} o HCO_3^-.

- Métodos que modifican A_T manteniendo DIC constante. Este grupo incluye la adición de ácidos fuertes y bases (generalmente HCL/NaOH); adición de CO_3^{2-} o HCO_3^- (sin ácido posterior. En este caso se mantiene el pH constante).

Otro método utilizado es la manipulación de la concentración de Ca^{2+}. Aunque no varía la química del carbono en sí, modifica el estado de saturación del carbonato cálcico. Por ello, ha sido utilizado en algunos casos para distintos estudios de calcificación (revisado en Gattuso *et al.* 2010).

El método elegido para llevar a cabo el estudio que ser recoge en el capítulo 5 de esta tesis fue la adicción de Na_2CO_3 y HCl. Hurd *et al.* (2009) propone que aquellos métodos en los que A_T se mantiene constante variando DIC (Fig. 11) simulan de forma más real la variación del sistema carbonato en el océano. De tal manera, estos deben ser los métodos de elección para el estudio de incorporación de carbono y tasas de crecimiento y fotosíntesis (Hurd *et al.* 2009).

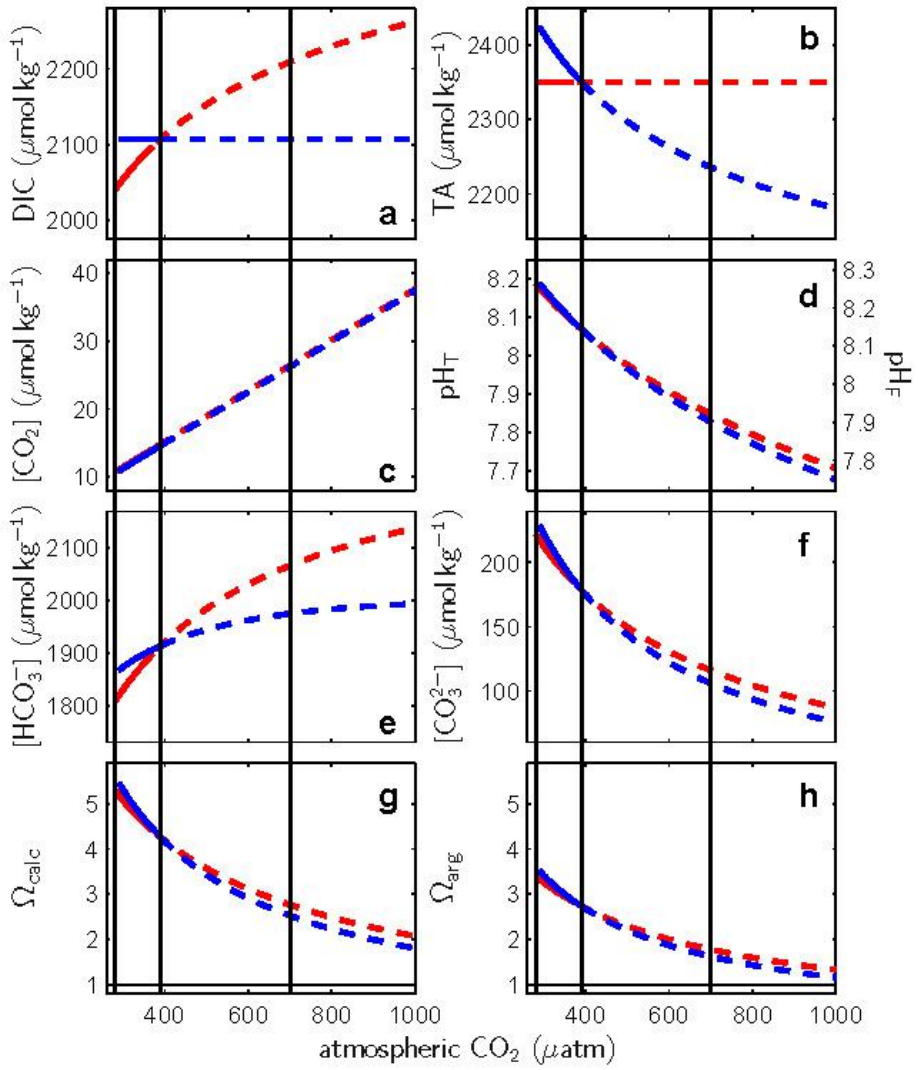


Fig. 10. Redistribución del sistema carbonato mediante dos tipos de manipulación: métodos que alteran DIC manteniendo A_T (TA) constante (rojo) o métodos que alteran A_T (TA) manteniendo DIC constante (azul). Fuente: Schulz *et al.* 2009.

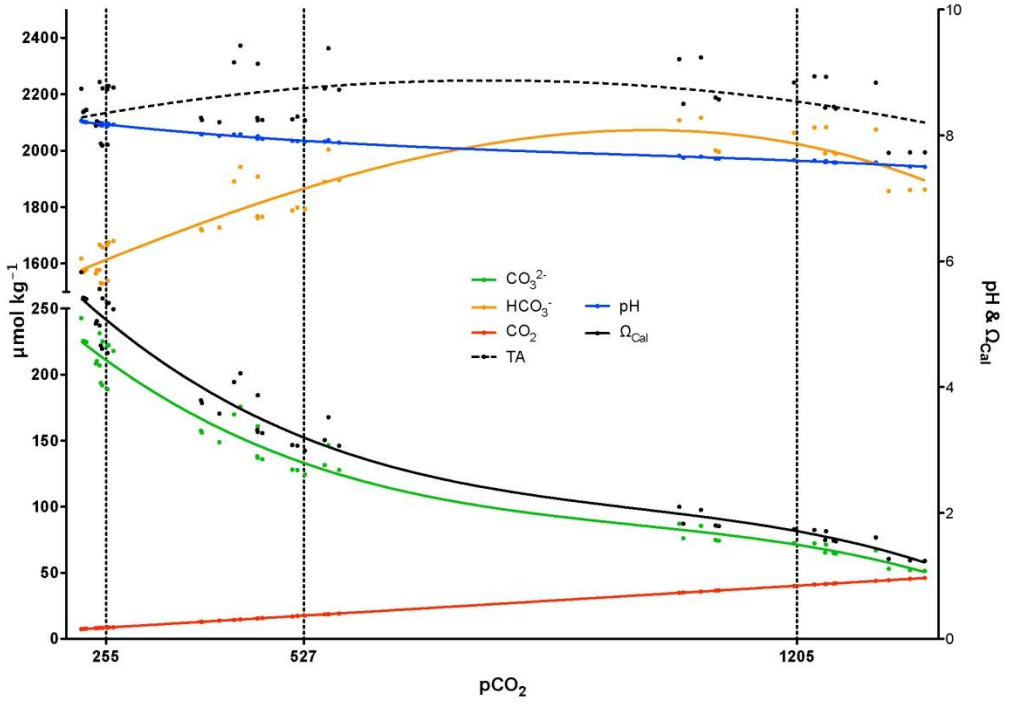


Fig. 11. Representación de la re-equilibración de los distintos componentes del sistema carbonato asociado al aumento de la presión parcial de CO_2 (pCO_2). Datos tomados para 255 ppm, 527 ppm y 1205 ppm. Datos obtenidos del experimento presentado en el siguiente capítulo.

VI. RESULTADOS

"Con números se puede demostrar cualquier cosa. "

(Thomas Carlyle. Historiador británico)

CAPITULO I

ESTRATEGIAS GENETICAS DE LA ADAPTACIÓN RÁPIDA A AMBIENTES NATURALES EXTREMOS

"*If one way be better than another, that you may be sure is nature's way*".

(Aristóteles)

Existen ambientes naturales caracterizados por valores extremos de pH, toxicidad, temperatura y otros factores que mantienen poblaciones de organismos viviendo a los límites de su tolerancia fisiológica (Seckbach y Oren 2007). Los organismos son capaces de sobrevivir en condiciones extremas a través de distintos mecanismos de adaptación. Si las condiciones ambientales no son demasiado extremas, el fitoplancton es capaz de sobrevivir por medio de la modificación de la expresión de sus genes (fenómeno de aclimatación) (Bradshaw y Hardwick 1989). En el caso de que los valores ambientales superaran los límites de aclimatación fisiológica de los organismos, los fenómenos de adaptación genética serían los únicos que asegurarían la supervivencia de la nueva especie (Sniegowski y Lenski 1995; Sniegowski 2005). Si las condiciones son demasiado extremas podría ocurrir que los organismos nunca llegaran a adaptarse.

Trabajos previos han demostrado que las algas eucariotas que habitan sus aguas podrían ser los descendientes de aquellos organismos que aseguraron su supervivencia a través de la selección de aquellos mutantes resistentes que surgieron al azar antes de entrar en contacto con las aguas hostiles (Flores-Moya *et al.* 2005; Sniegowski 2005; Costas *et al.* 2007). Los tres trabajos presentados en este capítulo pretenden ampliar el conocimiento sobre los procesos adaptativos del fitoplancton en este tipo de ambientes extremos. Los ambientes naturales extremos se pueden considerar, por tanto,

laboratorios naturales para el estudio de las distintas estrategias adaptativas de los organismos para sobrevivir y proliferar ante tales condiciones selectivas. Para ello se eligieron dos organismos mesófilos aislados de aguas no extremas, la clorofita *Dictyosphaerium chlorelloides* y la cianobacteria *Microcystis aeruginosa* como representantes de un organismo eucariota y procariota respectivamente. Para la determinación de la capacidad de adaptación de las distintas especies así como para la determinación del proceso adaptativo (mediante la selección de mutantes resistentes o adaptación fisiológica) se utilizó el Análisis de fluctuación como herramienta experimental. El capítulo se divide en tres trabajos examinando tres ambientes concretos:

- El primer trabajo utilizó como ejemplo de ambiente extremo el Arroyo de Aguas Agrias en Tharsis (al norte de la ciudad de Huelva), el cual presenta un pH ácido de 2.5 y contiene grandes concentraciones de metales tóxicos para el fitoplancton.
- El segundo trabajo examina la adaptación al agua de una laguna de la isla de Vulcano (en las Islas Aeolian, Italia) donde se encuentra el volcán Vulcano. Las aguas de las lagunas alrededor del volcán presentan valores extremos tanto de pH como de temperatura, presentando características idóneas como modelo para la adaptación del fitoplancton en un ambiente geotérmico.
- El último trabajo presenta también un sistema acuático de origen volcánico: El sistema Río Agrio-Lago Caviahüe (Argentina). Este sistema presenta un gradiente de toxicidad natural con aguas extremas con pH muy reducido en zonas altas y cercanas al volcán que van reduciendo sus características extremas según se va descendiendo en el sistema y va recibiendo aporte de otras masas de agua.

Los tres sistemas fueron considerados como modelos interesantes para el análisis de la adaptación de microalgas bajo distintos ambientes extremos. En el caso de los dos primeros artículos, la eucariota *Dictyosphaerium chlorelloides* fue capaz de adaptarse como resultado de la selección de mutantes resistentes que surgieron al azar antes de

entrar en contacto con el agua extrema. Las aguas del sistema Agrio Argentino presentan un perfecto ejemplo natural de la capacidad de adaptación del fitoplancton según sus límites de tolerancia bajo un gradiente natural extremo en el que nos encontramos desde fenómenos de adaptación fisiológica, genética, hasta la incapacidad de adaptación. La cianobacteria *Microcystis aeruginosa* no fue capaz de desarrollar resistencia para asegurar su adaptación en ninguna de las aguas examinadas.

I.I. Adaptation of the chlorophycean Dictyosphaerium chlorelloides to Stressful acidic, mine metal-rich waters as result of pre-selective mutations

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Adaptation of the chlorophycean *Dictyosphaerium chlorelloides* to stressful acidic, mine metal-rich waters as result of pre-selective mutations

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ABSTRACT

Several species of microalgae, closely related to mesophilic lineages, inhabit the extreme environment (pH 2.5, high levels of metals) of the Spain's Aguas Agrias Stream water (AASW). Consequently, AASW constitutes an interesting natural laboratory for analysis of adaptation by microalgae to extremely stressful conditions. To distinguish between the pre-selective or post-selective origin of adaptation processes allowing the existence of microalgae in AASW, a Luria–Delbrück fluctuation analysis was performed with the chlorophycean *Dictyosphaerium chlorelloides* isolated from non-acidic waters. In the analysis, AASW was used as selective factor. Preselective, resistant *D. chlorelloides* cells appeared with a frequency of 1.1×10^{-6} per cell per generation. AASW-resistant mutants, with a diminished Malthusian fitness, are maintained in non-extreme waters as the result of a balance between new AASW-resistant cells arising by mutation and AASW-resistant mutants eliminated by natural selection (equilibrium at *c.* 12 AASW-resistants per 10^7 wild-type cells). We propose that the microalgae inhabiting this stressful environment could be the descendents of chance mutants that arrived in the past or are even arriving at the present.

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1. Introduction

In extreme environments characterized by values of ecological factors exceeding the physiological limits of organisms, survival depends exclusively on adaptive evolution which occurs by selection on pre-existing genetic variation (Sniegowski and Lenski, 1995; Hughes, 1999; Sniegowski, 2005). Surprisingly, there are almost no studies that have made a direct connection between the rates of origin of favored mutants and the process of adaptation in diploid, multi-celled organisms living in well-defined populations. In contrast, many lineages of microalgae are haploid, single-celled and asexual, and their populations are composed of countless cells (Margulis and Schwartz, 1982). Consequently, these organisms are adequate to study the process of adaptation based on favored mutants. Moreover, there are few circumstances under which a specific mutation rate that is of relevance to adaptation can be measured.

The study of genetic adaptation of microalgae to extreme environments (i.e. characterized by extreme values of pH, toxics, and mineral excess) is an adequate approximation to the problem of the origin of favored mutants and the process of adaptation. Recently we demonstrated that the green alga *Spirogyra insignis* (Hassall) Kützing (Streptophyta) inhabiting the acidic (pH 4.1–4.5), sulphureous water from La Hedionda spa (S Spain) could be explained by selection of pre-selective mutants of mesophilic algal lineages inhabiting in non-acidic waters (Flores-Moya et al., 2005). Similarly, we suggested that eukaryotic microalgae, resistant to acidic (pH 1.7–2.5) and metal-rich waters from the Spain's Rio Tinto, arose randomly by rare spontaneous mutations and, as a result, algal populations could able to instantaneously adapt to Rio Tinto water by means of selection of resistant-mutants growing in non-extreme populations (Costas et al., 2007). An interesting question is to elucidate if adaptation to these stressful environments always take place rapidly through selection of resistant mutants or, in some cases, it could be the consequence of physiological adaptation (i.e. acclimation).

The aim of this work was to analyze adaptation of microalgae in the fascinating example of the extreme environment of Aguas Agrias Stream water (AASW) (meaning sour waters in English), located near the village of Tharsis (north of the city of Huelva,

Abbreviations: AASW, Aguas Agrias Stream water; F'_m, maximum fluorescence of light-adapted cells; F_t, steady-state fluorescence of light-adapted cells; m^r_{AASW}, Malthusian fitness parameter from AASW-resistant cells; m^s_{AASW}, Malthusian fitness parameter from AASW-sensitive cells; N_0, no. of cells at the start of the experiment; N_t, no. of cells at the end of the experiment; P_0, proportion of cultures without AASW-resistant cells in the set 1; q, frequency of AASW-resistant alleles in non-AASW-exposed populations; s, coefficient of selection; Φ_{PSII}, effective quantum yield; μ, mutation rate.

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SW Spain). AASW is very acid (pH 2.5), and contains high concentrations of metals (Fe at 1435 mg l^{-1}, Al at 551 mg l^{-1}, Zn at 324 mg l^{-1}, Mn at 143 mg l^{-1}, Cu at 64 mg l^{-1}, As(V) at 657 μg l^{-1} and Pb at 650 μg l^{-1}) (Sánchez-Rodas et al., 2006). Even in such extreme conditions, green patches of algae can be observed. To accomplish the objectives of the work, we initially performed a screening of microalgae species inhabiting AASW. Secondly, we studied if algal community inhabiting AASW could be explained as the result of selection of resistant mutants, or as result of other different adaptive process. For this purpose, a fluctuation analysis (Luria and Delbrück, 1943) was performed by using a wild-type strain of the mesophilic, cosmopolitan chlorophycean *Dictyosphaerium chlorelloides* (Naumann) Komarek and Perman isolated from a pristine, slightly alkaline (pH 8.0) high mountain lake from Sierra Nevada (S Spain) as experimental organism, and AASW as selective agent. This species rapidly adapt to grow in AASW via the action of natural selection on resistant mutants that appear spontaneously in wild-type populations. Thus, the presence of microalgae in the stressful environment of the AASW could be explained in accordance with the neo-Darwinian adaptive evolution hypothesis.

2. Materials and methods

2.1. Phytoplankton community in the AASW

Sampling of water and phytoplankton was carried out in May 2006, nearby Tharsis mine (37°35′28 N, 007°03′31 W), an area that has been exploited in ancient times and during the last two centuries until the 1990s. The sample point was located within the so-called Iberian Pyrite Belt, a volcanogenic massive sulfide province (Sáez et al., 1999). AASW is heavily loaded in mine drainage due to the lixiviates from the Tharsis mine (Checkland, 1967). Three AASW samples of 5 l were collected and homogeneously mixed. The resulting integrated water sample was filtered (0.22 μm, Stericup, Millipore Co., Billerica, MA, USA).

Phytoplankton was identified in fresh samples (directly after collection) using a McArthur portable microscope (Kirk Technology, England), and counted on fixed samples (4% PBS-buffered formalin) in settling chambers using an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany). Identification of algae was carried out in accordance with Cox (1996) for diatoms, Wolowski (2002) for euglenophytes, and John and Tsarenko (2002), Johnson (2002) and Pentecost (2002) for green algae.

2.2. Experimental organism

A wild-type strain of *D. chlorelloides* from the Algal Culture Collection of the Faculty of Veterinary, Complutense University (Madrid, Spain) was grown in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, at 20 °C. The strain was isolated from a pristine, slightly alkaline (pH 7.8–8.0) high mountain lake from Sierra Nevada (S Spain). Cultures were maintained axenically in mid-log exponential growth (Cooper, 1991) by serial transfers of subcultures to fresh medium. Only cultures without detectable bacteria were used in the experiments. Although *D. chlorelloides* forms 2- or 4-celled, rarely 16-celled colonies, and is capable of sexual reproduction in the nature (John and Tsarenko, 2002), the strain tested here exclusively propagated by asexual reproduction, and it was represented by single-celled individuals. Prior to the experiments, the culture was cloned (by isolating a single cell) to avoid including any previous spontaneous mutants that accumulated previously.

2.3. Toxicity test: effect of AASW on Malthusian fitness and effective quantum yield

With the purpose to test the toxic effect of the AASW, we measured the changes in Malthusian fitness (m) and effective quantum yield from photosystem II (Φ_{PSII}) when the wild-type strains of *D. chlorelloides* were cultured in AASW.

Culture samples (5×10^5 cells) from mid-log exponentially growing cultures of *D. chlorelloides* wild-type strain were placed in experimental tubes containing 1.5 ml of AASW. Controls in BG-11 medium were also prepared. Malthusian fitness values were calculated in three replicates in AASW as well as in three controls, using the equation from Crow and Kimura (1970):

$$m = \mathrm{Log_e}(N_t/N_0)/t \quad (1)$$

where t = 5 d, $N_0 = 5 \times 10^5$ cells, and N_t is the cell number at the end of the experiment. Experiments and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer.

The effective quantum yield (Φ_{PSII}) was also measured in triplicates of experiments and controls using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) at five different time points (1, 12, 24, 48, and 72 h). Effective quantum yield was calculated as follows:

$$\Phi_{PSII} = (F'_m - F_t)/F'_m \quad (2)$$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al., 1986; Maxwell and Johnson, 2000).

2.4. Fluctuation analysis of AASW-sensitive → AASW-resistant transformation

A Luria and Delbrück (1943) fluctuation analysis was carried out as described in Fig. 1. Two different sets of experimental cultures were prepared. In the set 1 experiment, 95 test tubes were inoculated with $N_0 = 10^2$ cells of *D. chlorelloides* wild-type strain (a number small enough to reasonably ensure the absence of pre-existing mutants in the strain). Cultures were grown in 10 ml BG-11 medium until $N_t = 1.04 \times 10^5$ cells and afterwards exposed to AASW. For this purpose, the cultures were centrifuged to form a pellet of cells in the tube, the medium was decanted, and 10 ml of AASW was added to the tubes. In the set 2 controls, 20 aliquots of 1.04×10^5 cells from the same parental population (growing in 10 ml BG-11 medium) were separately transferred to test tubes containing AASW. Cultures were observed for 80 d (thereby insuring that one mutant cell could generate enough progeny to be detected), and the resistant cells in each culture (both in set 1 and set 2) were counted. The cell count was performed by at least two independent observers.

Two different results can be found in the set 1 experiment, each of them being interpreted as the consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1), the variance in the number of cells per culture would be low if resistant cells arose during the exposure to the selective agent (i.e. by physiological adaptation). Because every cell is likely to have the same chance of developing resistance, inter-culture (test tube-to-test tube) variation would be consistent with the Poisson model (i.e. variance/mean = 1). On the contrary, if high variation in the inter-culture number of resistant cells is found (i.e. variance/mean > 1), it means that resistant cells appeared by rare spontaneous mutations occurring before AASW exposure (that is to say, they occurred during the time in which the cultures grew to N_t from N_0 cells, before the exposure to AASW, Fig. 1, set 1B). Therefore, the test tube-to-test tube variation would not be consistent with the Poisson model.

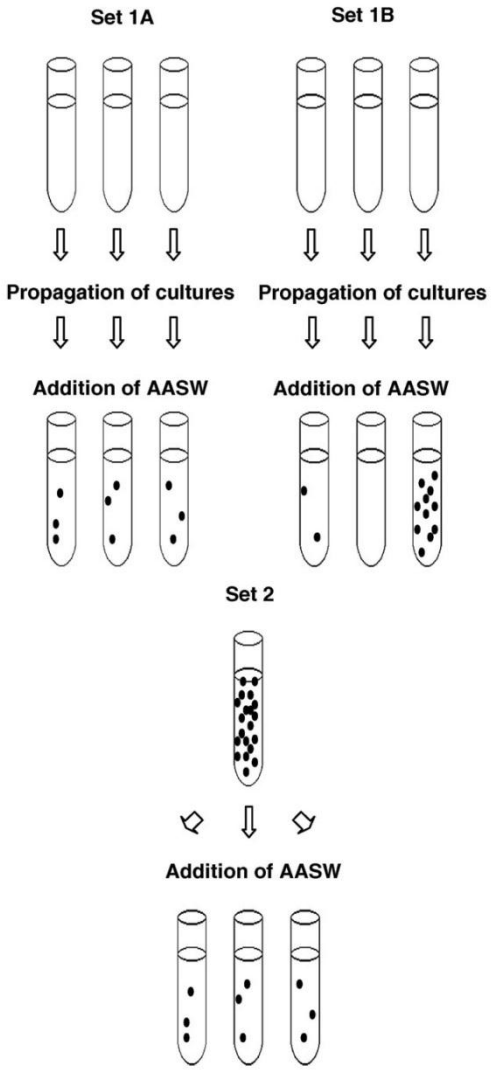

Fig. 1. Schematic diagram of possible results obtained in the experiment (modified from the classic Luria and Delbrück fluctuation analysis). Set 1: different cultures of *Dictyosphaerium chlorelloides* (each started from a small inoculum, $N_0 = 10^2$ cells) were propagated under non-selective conditions (i.e. BG-11 medium) until a very high cell density was reached ($N_t = 1.04 \times 10^5$ cells), and then transferred to the selective agent (i.e. AASW). If resistant cells arose during the exposure to AASW (physiological adaptation or post-adaptive mutations), the number of resistant cells in all the cultures must be similar (set 1A). If resistant cells arose by rare mutations occurring in the period of the propagation of cultures (i.e. before exposure to AASW) the number of resistant cells in all the cultures must be different (set 1B). In the figure, one mutational event occurred late in the propagation of culture 1 (therefore, the density of AASW-resistant cells found is low) and early in the propagation of culture 3 (thus, density of AASW-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. Set 2: Different replicates from the same parental culture sampling the variance of the parental population are used as an experimental control. In this case, the number of resistant cells in all the cultures must be similar.

Set 2 is the experimental control of the fluctuation analysis (Fig. 1). Either way resistance appears, variance is expected to be low, because set 2 samples the variance of the parental population. If a different variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells appeared by rare spontaneous mutations that occurred before exposure to the selective agent. If a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells appeared after exposure to the selective agent (i.e. by acclimation).

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. Due to the methodological limitations imposed by a fluctuation analysis using liquid cultures of algae, the proportion of cultures of set 1 showing no resistant mutants (P_0 estimator; Luria and Delbrück, 1943) after AASW exposure was the parameter used to calculate the mutation rate (μ) as follows:

$$\mu = -\mathrm{Log}_e P_0/(N_t - N_0) \quad (3)$$

2.5. Mutation–selection equilibrium

If the AASW-sensitive → AASW-resistance mutation is recurrent, and the mutant allele is detrimental to fitness in the absence of AASW, then new resistant alleles arise in each generation by mutation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance. As a result, at any one time there will be a certain number of resistant cells that are not yet eliminated. The average number of such mutants will be determined by the balance between μ and the rate of selective elimination (s), in accordance with Kimura and Maruyama (1966):

$$q = \mu/(\mu + s) \quad (4)$$

where q is the frequency of the AASW-resistant allele and s is the coefficient of selection against this resistant allele, calculated as follows:

$$s = 1 - (m^r_{AASW}/m^s_{AASW}) \quad (5)$$

where m^r_{AASW} and m^s_{AASW} are the fitness of AASW-resistant and AASW-sensitive cells measured in non-selective conditions (i.e. in BG-11 culture medium), respectively.

3. Results

The phytoplankton community of AASW in May 2006 was composed by diatoms, chlorophyceans and euglenophyceans (Table 1); cyanobacteria were not detected.

The toxic effect of AASW on Φ_{PSII} and m from *D. chlorelloides* wild-type strain cells was very dramatic: Φ_{PSII} was completely inhibited (100% inhibition with respect to unexposed controls) at all the time points tested (from 1 to 72 h). Similarly, a value of $m = 0$ was found in AASW exposed cultures; in contrast, the Malthusian fitness value in the controls (m^s_{AASW}) was 0.600 ± 0.011 doublings d^{-1} ($n = 3$).

Table 1
Most abundant microalgal species (cells ml^{-1} in single-celled species or filaments ml^{-1} in filamentous species, mean ± SD, $n = 5$) detected in AASW in May 2006

Phylum	Species	Abundance
Bacillariophyta	*Nitzschia acicularis* (Kützing) W. Smith	438 ± 41
	Navicula exigua Gregory	92 ± 38
	Fragilaria sp.	78 ± 34
Streptophyta	*Spirogyra* sp.	415 ± 65
Chlorophyta	*Chlorella* sp.	310 ± 56
	Oocystis borgei J. Snow	40 ± 3
	Chlamydomonas sp.	17 ± 11
	Scenedesmus arcuatus Lemmermann	8 ± 4
Euglenophyta	*Euglena* sp.	19 ± 11
	Trachelomonas granulosa Playfair	7 ± 3

Table 2
Fluctuation analysis of AASW-sensitive → AASW-resistance transformation in *Dictyosphaerium chlorelloides*

	Set 1	Set 2
No. of replicate cultures	95	20
No. of cultures containing the following no. of AASW resistant cells:		
0	85	0
$1–10^3$	4	0
$10^3–10^4$	3	0
$>10^4$	3	20
Variance/mean (of the no. of resistant cells per replicate)	153.1	0.9
μ (mutants per cell per generation)	1.1×10^{-6}	

When conducting the fluctuation analysis, first of all the cell density was drastically reduced in each experimental culture of sets 1 and 2 due to massive destruction of sensitive cells. However, after further incubation for several weeks, some cultures increased in density again, apparently due to growth of a AASW-resistant variant. Ten of the initial 95 cultures in the set 1 recovered after 80 d under AASW exposure (Table 2). By contrast, every set 2 culture recovered, and AASW-resistant cells were detected in all cultures (Table 2). A high fluctuation was detected in set 1 cultures; the variance significantly exceeded the mean (variance/mean $\gg 1$; $P < 0.001$, using χ^2 as a test of goodness of fit). In contrast, almost null fluctuation was observed in set 2 (variance/mean ≈ 1, consistent with Poisson variability; $P < 0.05$, using χ^2 as a test of goodness of fit) (Table 2). Consequently, the high fluctuation found in set 1 cultures should be due to processes other than sampling error, and it could be inferred that AASW-resistant cells arose prior to AASW exposure by rare, spontaneous mutations rather than by specific adaptation (i.e. acclimation) during AASW exposure. The estimated μ value for AASW-sensitive → AASW-resistance transformation in *D. chlorelloides* was of 1.1×10^{-6} mutants per cell per generation (Table 2).

AASW-resistant mutants growing in BG-11 medium (i.e. in absence of the selective agent) showed a Malthusian fitness value (m^r_{AASW}) of 0.048 ± 0.003 doublings d^{-1}. The overall mean values of m^s_{AASW} and m^r_{AASW} measured in non-selective conditions (0.600 and 0.048 doublings d^{-1}, respectively) were used to estimate the coefficient of selection of AASW-resistant mutants ($s = 0.92$). By using the values of μ and s, the frequency of AASW-resistant allele was calculated: *c.* 12 *D. chlorelloides* AASW-resistant mutants per 10^7 cells as the consequence of the balance between mutation and selection.

4. Discussion

An astonishing diversity of eukaryotic microalgae, closely related to neutrophilic lineages, inhabit the acidic and metal-rich waters from Aguas Agrias Stream (see Table 1). This result is comparable to algal sampling carried out in the similar environment from Spain's Rio Tinto (also located in the Iberian Pyrite Belt), where green algae and euglenophytes closely related to their corresponding neutrophilic lineages have been detected both by molecular, cultivation-independent techniques (Amaral Zettler et al., 2002) and direct isolation (Costas et al., 2007) samplings. However, the catastrophic effect of AASW on m and Φ_{PSII} of *D. chlorelloides* isolated from non-extreme waters suggests that the survival of this species in AASW could only be achieved by some kind of adaptation. The key to understanding adaptation of this species to the extreme environment of the AASW is to examine the rare algal variants that occur after the massive destruction of the sensitive cells by AASW.

The fluctuation analysis allows us to distinguish between cells that became resistant to AASW from acquired specific resistance (i.e. physiological adaptation) and cells resistant due to spontaneous mutations that occur randomly during propagation of organisms prior to exposure to AASW (Luria and Delbrück, 1943). The large fluctuation in number of AASW-resistant cells observed in set 1 experiments, in contrast with the no fluctuation in set 2, unequivocally demonstrates that resistant cells arose by rare spontaneous mutation (AASW did not stimulate the appearance of resistant cells at all) and not through direct and specific adaptation in response to AASW. It should be noted that it is impossible to detect post-selective mutations (adaptive mutations) if the rate of these kinds of mutations for AASW-resistance were smaller than 10^{-8} using fluctuation analysis. Despite this, the rapid lethal effect of AASW seems unlikely to allow the appearance of adaptive mutations, which are observed in non-proliferating microbial populations after being incubated on non-lethal selective medium plates (Cairns et al., 1988; Foster, 2000).

Evolutionary biology is often caricatured as a strictly descriptive science. However, here we show that evolutionary change in microalgae can be studied on short time scales with a robust experimental approach such as the Luria–Delbrück fluctuation analysis. Moreover, we followed a similar approach to study adaptation to stressful lethal conditions (originated by anthropogenic contamination, or extreme natural environments) in cyanobacteria and microalgae; in all the cases, the adaptation was achieved as the result of a rare event: the spontaneous mutation from sensitivity to resistance (with frequencies of 1 resistant mutant cell per 10^5–10^7 wild-type cells) that occurs randomly prior to the cells coming into contact with the selective agent (Costas et al., 2001; López-Rodas et al., 2001; Baos et al., 2002; García-Villada et al., 2002, 2004; Flores-Moya et al., 2005; Costas et al., 2007; López-Rodas et al., 2007, 2008). If it is assumed that this is true under any stressful lethal conditions, it could be hypothesized that future changes in the biosphere, as consequence of Global Change, are not a compromise for survival of cyanobacteria and microalgae because they can develop genetic adaptation and, simultaneously, their populations are composed by countless cells. Thus, the probability to survive to sudden stressful changes could be enough high to avoid any future environmental crisis, but with lower values of productivity (Costas et al., 2007; López-Rodas et al., 2007), and the arising of morphological novelties (López-Rodas et al., 2006, 2007). The only vital limit that could not be avoided is acidification of waters in the case of cyanobacteria. These organisms have been reported to be absent from moderately or extremely acidic environments (Brock, 1973; Knoll and Bauld, 1989; Albertano, 1995; Gimmler, 2001), and a pH limit of 4.8 was postulated for proliferation of cyanobacteria (Brock, 1973). In fact, we did not detect cyanobacteria in the phytoplankton sampling of AASW. Moreover, in previous fluctuation experiments with the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann, using as selective agent acid waters from Spain's Rio Tinto (Costas et al., 2007), adaptation was not detected.

Summarizing, eukaryotes rarely flourish in extreme environments; however, we propose that these environments could be colonized by mesophilic organisms inhabiting non-extreme environments by neo-Darwinian adaptive evolution.

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I.II. Living in Vulcan´s forge: Algal adaptation to stressful geothermal ponds on Vulcano Island (southern Italy) as a result of pre-selective mutations.

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Living in Vulcan's forge: Algal adaptation to stressful geothermal ponds on Vulcano Island (southern Italy) as a result of pre-selective mutations

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SUMMARY

Four species of eukaryotic algae proliferate in the sulfureous, acidic (pH 3.1) water of the largest geothermal pond on Vulcano Island (southern Italy). Consequently, this pond constitutes a natural laboratory for analysis of adaptation by phytoplankters to extremely stressful conditions. To distinguish between the pre-selective or post-selective origin of adaptation processes allowing the existence of phytoplankters in the pond, a Luria-Delbrück fluctuation test was carried out with the chlorophycean *Dictyosphaerium chlorelloides* and the cyanobacterium *Microcystis aeruginosa*, both isolated from non-extreme waters; natural water from the Vulcano Island pond was used as selective factor. Pre-selective, resistant *D. chlorelloides* cells appeared with a frequency of 4.7×10^{-7} per cell per generation. We propose that the micro-algae inhabiting this stressful pond could be the descendents of chance mutants that arrived in the past or are even arriving at the present. The genetic adaptation of *D. chlorelloides* to Vulcano waters could help to explain the survival of photosynthesizers in very stressful geothermal waters during the Neoproterozoic 'snowball Earth', a period when primary production collapsed in the biosphere. On the other hand, adaptation to these conditions was not observed in *M. aeruginosa*, suggesting that cyanobacteria may not be able to develop any kind of adaptation to Vulcano pond water.

Key words: adaptive evolution, cyanobacteria, fluctuation analysis, micro-algae, 'snowball Earth', Vulcano Island.

INTRODUCTION

Different aspects of the interactions of extreme environmental conditions from geothermal waters and the micro-organisms that inhabit these environments have been previously addressed (Brock 1978; Setter 1996; Madigan & Narrs 1997; Horikoshi & Grant 1998; Elster *et al.* 2001; Rothschild & Mancinelli 2001; Newman & Banfield 2002; Seckbach 2007). However, in the case of mesophilic micro-algae and cyanobacteria inhabiting geothermal ponds, a question yet remains open: how has it achieved the adaptation to survive and proliferate under stressful conditions?

If the stressful conditions do not exceed the limits of the physiological tolerance of algae, the survival is the result of physiological adaptation (i.e. acclimation) supported by modifications of gene expression (Bradshaw & Hardwick 1989). On the other hand, in extreme environments characterized by values of ecological factors exceeding the physiological limits of algae, survival depends exclusively on adaptive evolution, which results from the occurrence of new mutations that confer resistance (Sniegowski & Lenski 1995; Flores-Moya *et al.* 2005; Sniegowski 2005; Costas *et al.* 2007; López-Rodas *et al.* 2008a,b). However, recent evolutionary studies of bacteria have suggested that hypothetical adaptive mutations could be a process resembling Lamarckism, which, in the absence of lethal selection, produces mutations that relieve selective pressure (Cairns *et al.* 1988; Foster 2000). Examples of adaptive mutations or related phenomena have also been recognized in yeast (Heidenreich 2007) but, as far as we know, they have not been reported in other micro-organisms such as cyanobacteria and micro-algae. Therefore, the key to resolving this debate is to know the pre-selective or post-selective origin of new mutations. Surprisingly, there are almost no studies of

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the origin of favored mutants and their survival beyond physiological limits in diploid, multi-celled organisms living in well-defined populations (Sniegowski 2005). In contrast, cyanobacteria and many lineages of micro-algae are haploid, single-celled and asexual, and their populations are composed of countless cells (Margulis & Schwartz 1982). Consequently, these organisms are adequate to study the process of adaptation based on favored mutations.

Vulcano Island (Aeolian Islands, southern Italy) provides an excellent model to study adaptation of phytoplankters to stressful geothermal ponds that are lethal for mesophilic lineages. It contains a main volcano (named Vulcano) and several overlapping minor volcanic centers (Keller 1980). Vulcano is in a sense the most famous volcano in the world: Vulcan, the Roman god of fire and metalworking, responsible for making the weapons of the gods, made his home inside the volcano. The main cone originated sometime after 11 000–8500 years ago (Frazzetta *et al.* 1984). At least four volcanic cycles contributed to the formation of Vulcano Island (Cortese *et al.* 1986). In addition, the last eruption of Vulcano (from 1888 to 1890) deposited 5 m of pyroclastic material (Cortese *et al.* 1986) and indubitably killed all of the phytoplankton of the ponds on the island.

The aim of this work was to evaluate, from an evolutionary point of view, adaptation of cyanobacteria and micro-algae, to growth and survival in the stressful environment of a pond on Vulcano Island. For this purpose, we first carried out a survey of phytoplankton inhabiting Vulcano pond water (VPW; the largest pond in the island was selected). Because we found few algal species closely related to mesophilic ones, we then carried out a fluctuation analysis (Luria & Delbrück 1943) on a mesophilic chlorophycean using VPW as selective agent; we also tested the possible adaptation to VPW by a cyanobacterium. The fluctuation analysis is a statistical and experimental procedure, which allows us to distinguish between cells that become resistant from acquired specific adaptation (including both physiological adaptation or acclimation, and possible adaptive mutations; the first case is not an evolutionary event) and resistant cells arising from spontaneous mutations that occur randomly during propagation prior to exposure to the selective agent. Although fluctuation analysis has been proved to be the most sensitive approach to distinguish between rare spontaneous mutation and direct adaptation to environmental selection (Cole *et al.* 1976), the study of mutation rates in groups other than bacteria has been hampered by certain cumbersome aspects of the analysis (Rossman *et al.* 1995).

This study is a complement to other work carried out in a framework focused on understanding phytoplankton adaptation to extreme natural conditions (Flores-Moya *et al.* 2005; Costas *et al.* 2007; López-Rodas *et al.* 2008a,b). In this way, here we show that survival and proliferation of phytoplankters in an extreme natural pond on Vulcano Island could be achieved as a result of rare spontaneous mutations at one or a small number of loci.

MATERIALS AND METHODS

Environmental conditions and phytoplankton community in the Isthmus pond of Vulcano Island

Sampling of water and phytoplankton was carried out in July 2006; for this purpose, the largest pond on the island (around 70 m^2 surface $\times$ 0.15 m deep) located in the periphery of Vulcano Island (named Isthmus pond; 08°25′N, 014°57′E) was selected. The values of pH and temperature in the pond were determined by using a YSI 6820-C-M probe (Yellow Springs, OH, USA). In order to determine sulfide levels, four drops of Zn $(CH_3CO_2)_2$ 2 N were added to a 100 mL water sample and the sample bottle was immediately sealed, excluding any air. The sample was stored at 4°C in darkness until sulfide determination by titration with $Na_2S_2O_3$ 0.1 N, in accordance with APHA-AWWA-WPCF (1992). In addition, five 1 L VPW samples were collected and homogeneously mixed. The resulting integrated water sample was filtered (0.22 μm, Stericup, Millipore Co., Billerica, MA, USA) and kept in a closed bottle excluding any air, stored at 4°C in darkness until the laboratory experiments (toxicity tests and fluctuation analysis) were carried out.

Phytoplankton was identified in fresh samples (directly after collection) using a McArthur portable microscope (Kirk Technology, Cambridge, UK), and counted on fixed samples (4% formalin) in settling chambers using an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany). Identification of algae was carried out in accordance with Cox (1996) for diatoms, Wolowski (2002) for euglenophytes, and John and Tsarenko (2002) and Pentecost (2002) for green algae.

Experimental organisms and culture conditions

A wild-type strain of the chlorophycean *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman was isolated from a pristine, cool-temperate (5–10°C), non-sulfureous, slightly alkaline (pH 7.8–8.0) high mountain lake from Sierra Nevada (southern Spain); a strain of the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann was isolated from a pristine pond of non-acidic waters (pH 8.1) in Doñana National Park (southwest Spain). Both wild-type strains

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are deposited in the Algal Culture Collection of the Veterinary Faculty of Complutense University (Madrid, Spain). These strains were grown in 100 mL cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL BG-11 medium (Sigma-Aldrich Chemie, Taufkirchen, Germany), at 22°C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Although *D. chlorelloides* forms 2- or 4-celled (rarely 16-celled) colonies and is capable of sexual reproduction in nature (John & Tsarenko 2002), the strain was exclusively propagated by asexual reproduction, and it was represented by single-celled individuals. Cultures were axenically maintained in mid-log exponential growth (Cooper 1991) by serial transfers of subcultures to fresh medium, and only cultures without detectable bacteria were used in the experiments. Prior to the experiments, the cultures were cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the culture.

Toxicity test: effect of VPW on acclimated maximal growth rate

In order to test the toxic effect of the VPW, we measured the changes in acclimated maximal growth rate (m), in mid-log exponentially growing cells, from both wild-type strains cultured in this selective agent. Acclimated maximal growth rate is the Malthusian parameter of fitness under conditions of r selection (Crow & Kimura 1970; Spiess 1989). Culture samples (5.0×10.0^5 cells) from a mid-log exponentially growing culture of both species were placed in experimental tubes containing 1.5 mL of VPW at 30°C. Controls in BG-11 medium at 22°C were also prepared. The value of m was calculated in three replicates of each species in VPW as well as in three BG-11 medium controls, using the equation:

$$m = \log_e(N_t/N_0)/t \quad (1)$$

where t = 3 d, and N_0 and N_t are the cell numbers at the start and at the end of the experiment, respectively. Experiments and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer. The number of samples in each case was determined using the progressive mean procedure (Williams 1977), which assured a counting error <5%.

Fluctuation analysis of VPW-sensitivity to VPW-resistance transformation

A fluctuation analysis (Luria & Delbrück 1943) was carried out to discriminate between acquired adaptation (acclimation, and possible adaptive mutations) and pre-selective adaptation to VPW in *D. chlorelloides* and *M. aeruginosa* (see Fig. 1). In short, two different sets of experimental cultures were prepared. In the set 1 experiment, 70 culture flasks were inoculated with $N_0 = 10^2$ cells of *D. chlorelloides* (a number small

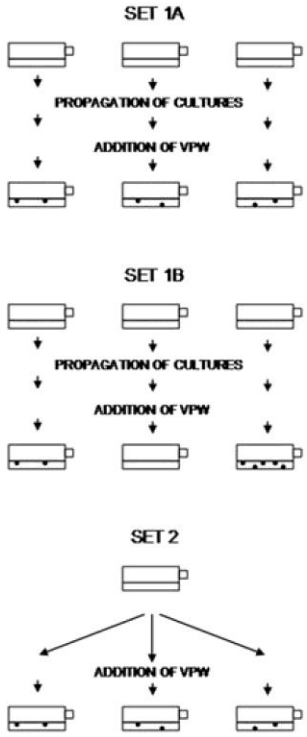

Fig. 1. Schematic diagram of possible results obtained in the experiment (modified from the classic Luria and Delbrück fluctuation analysis). In the set 1 experiment, different cultures (each started from a small inoculum, $N_0 = 10^2$ cells in both the chlorophycean *Dictyosphaerium chlorelloides* and the cyanobacterium *Microcystis aeruginosa*) were propagated under non-selective conditions (i.e. BG-11 medium, 22°C) until a very high cell density ($N_t = 9.5 \times 10^4$ cells in *D. chlorelloides*, and 5.0×10^5 in *M. aeruginosa*) was reached, and then transferred to Vulcano pond water (VPW) at 30°C. Set 1A: resistant cells arose during the exposure to the selective agent (physiological acclimation or adaptive mutations). In this case, the number of resistant cells in all of the cultures must be similar. Set 1B: adaptation by rare mutations occurring in the period of the propagation of cultures, that is, before exposure to the selective agent. One mutational event occurred late in the propagation of culture 1 (therefore, the density of VPW-resistant cells found was low) and early in the propagation of culture 3 (thus, density of VPW-resistant cells found was higher than in culture 1); no mutational events occurred in culture 2. In this case, the number of resistant cells in all of the cultures must be different. Set 2 samples the variance of parental populations as an experimental control. In this case, the number of resistant cells in all of the cultures must be similar.

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enough to reasonably ensure the absence of pre-existing mutants in the strain); 96 culture flasks were also inoculated with $N_0 = 10^2$ cells of *M. aeruginosa*. Cultures were grown in 20 mL of BG-11 medium at 22°C until $N_t = 9.5 \times 10^4$ cells in *D. chlorelloides* or $N_t = 2 \times 10^5$ cells in *M. aeruginosa*, and afterwards exposed to VPW at 30°C. For this purpose, the cultures were centrifuged to form a pellet of cells, the medium was decanted, and 20 mL of VPW was added to the flasks. For the set 2 control, 20 aliquots of 9.5×10^4 cells of *D. chlorelloides*, and 25 aliquots 2×10^5 cells in *M. aeruginosa*, from the same parental populations growing in 20 mL BG-11 medium at 22°C were separately transferred to culture flasks containing VPW at 30°C. Cultures were observed for 60 days (thus ensuring that one mutant cell could generate enough progeny to be detected), and the resistant cells in each culture were counted by at least three independent observers.

Two different results can be found in the set 1 experiment when conducting a fluctuation analysis, each of them being interpreted as the independent consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1A) the variance in the number of cells per culture would be low if resistant cells arose during the exposure to the selective agent (i.e. by acclimation or adaptive mutations). Because every cell is likely to have the same chance of developing resistance, inter-culture (flask-to-flask) variation would be consistent with the Poisson model (i.e. variance/mean ≈1). By contrast, if high variation in the inter-culture number of resistant cells is found (i.e. variance/mean >1), it means that resistant cells appeared by selection of rare spontaneous mutations occurring before selection, and the flask-to-flask variation would not be consistent with the Poisson model (that is to say, they occurred during the time in which the cultures grew to N_t from N_0 cells, before the exposure to VPW, Fig. 1, set 1B). Obviously, another result (0 resistant cells in each culture) could also be found, indicating that neither selection on spontaneous mutations that occur prior to VPW exposure, nor specific adaptation during the exposure to VPW, took place.

The set 2 cultures is the experimental control of the fluctuation analysis (Fig. 1). Variance is expected to be low, because set 2 samples the variance of the parental population. Thus, if a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells arose during the exposure to the VPW (by acclimation or adaptive mutations). In contrast, if the variance/mean ratio of set 1 is significantly greater than the variance/mean ratio of set 2 (fluctuation), this confirms that resistant cells arose by rare mutations that occurred before exposure to the VPW.

The fluctuation analysis also allows estimation of the rate of appearance of resistant cells. Because of the methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of cultures of set 1 showing non-resistant cells after VPW (i.e. the first term of the Poisson distribution, named the P_0 estimator; Luria and Delbrück (1943) was the parameter used to calculate the mutation rate (μ) as:

$$\mu = -\log_e P_0/(N_t - N_0) \qquad (2)$$

RESULTS

The shallow pond on Vulcano Island selected for our study is characterized by high sulfide levels (1.84 ± 0.10 mg L^{-1}), and acidic (pH 3.1 ± 0.1) and relatively warm (30.3 ± 0.5°C) conditions.

The phytoplankton flora in the pond is very poor: only four species of eukaryotic micro-algae were found. The dominant species were the chlorophyceans *Chlamydomonas variabilis* P. A. Dangeard and *Dictyosphaerium ehrenbergianum* Nägeli, with densities of 822 ± 193 and 773 ± 99 cells mL^{-1} ($n = 7$), respectively. The other two species were a centric diatom from the genus *Stephanodiscus* (cell density at 72 ± 26 per mL, $n = 7$), and the euglenophyte *Euglena* sp. (cell density at 62 ± 18 per mL, $n = 7$). Cyanobacteria were not detected in the pond.

The growth rate of the wild-type strains of *M. aeruginosa* and *D. chlorelloides* was inhibited ($m = 0$ doublings d^{-1} in both species) when they were cultured in filtered VPW at 30°C. In contrast, controls cultured in BG-11 medium at 22°C proliferated ($m = 0.68 \pm 0.04$ doublings d^{-1} for *D. chlorelloides*, and 0.53 ± 0.03 doublings d^{-1} for *M. aeruginosa*; $n = 3$ in both species).

When conducting the fluctuation analysis, the cell density of *D. chlorelloides* was drastically reduced in each experimental culture due to destruction of wild-type cells, sensitive to the VPW. However, after further incubation for 2 months, some *D. chlorelloides* cultures increased in density again, apparently due to growth of a VPW-resistant variant. In the case of set 1, three cultures recovered after 60 days under VPW exposure (Table 1). By contrast, every set 2 culture recovered, indicating the presence of VPW-resistant cells in all cultures (Table 1). A high fluctuation in the set 1 experiment (from 0 to 753 960 resistant cells per culture flask) was found (Table 1), exceeding significantly the variance of the number of resistant cells to the mean ($P < 0.001$, using χ^2 as a test of goodness of fit). The fluctuation observed was not a consequence of experimental error in sampling VPW-resistant cells because the analyses of set 2 showed low fluctuation in the number of VPW-resistant cells per flask (22 258 ± 1335). In fact, the ratio variance/mean of the no. of resistant cells per replicate in set 2 (see Table 1) was consistent with Poisson variability (i.e. variance/mean *c.* 1; $P < 0.05$, using χ^2 as a test of

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Table 1. Fluctuation analysis of Vulcano pond water (VPW)-resistant variants in wild-type strains of the chlorophycean *Dictyosphaerium chlorelloides* and the cyanobacterium *Microcystis aeruginosa*

	Set 1	Set 2
Dictyosphaerium chlorelloides (Chlorophyceae)		
No. cultures	70	20
No. cultures containing the following no. of VPW-resistant cells:		
0	67	0
$<10^3$	1	0
10^3–10^4	0	0
10^4–10^5	1	20
$>10^5$	1	0
Variance/mean (of the no. VPW-resistant cells per replicate)	>100†	1.3
μ (mutants per cell per generation)	4.7×10^{-7}	
Microcystis aeruginosa (Cyanobacteria)		
No. cultures	96	25
No. cultures containing VPW-resistant cells:	0	0

†Variance/mean >1; $P < 0.001$, using χ^2 as a test of goodness of fit.

goodness of fit). Consequently, we infer that VPW-resistant cells arose by rare, pre-selective spontaneous mutations prior to VPW exposure. The estimated μ of VPW-sensitive to VPW-resistant in *D. chlorelloides* was 4.7×10^{-7} mutants per cell per generation.

The cyanobacterium *M. aeruginosa* seemed to be unable to adapt to VPW (Table 1). Neither selection on spontaneous mutations that occur prior to VPW exposure, nor specific adaptation during the exposure to VPW, was enough to allow adaptation of *M. aeruginosa* to VPW.

DISCUSSION

Only four species of eukaryotic algae were found in the extreme environment of the Vulcano Island pond, suggesting that they might well have undergone adaptation to these hostile conditions. Moreover, the catastrophic effect of VPW on *D. chlorelloides* isolated from a more moderate habitat suggests that the survival of micro-algae in VPW could only be achieved by some kind of adaptation.

When *D. chlorelloides* was cultured in VPW, cultures became clear after a few days due to the massive destruction of the sensitive cells by the toxic effect of VPW. However, after further incubation for 2 months, some cultures became colored again, due to the growth of cells that were resistant to the toxic effect of VPW. The key to understanding adaptation of *D. chlorelloides* to the extremely adverse conditions of the VPW is to analyze the rare variants that proliferate after the massive destruction of the sensitive cells by this selective agent.

The large fluctuation in number of VPW-resistant cells observed in the set 1 experiment, in contrast to the insignificant fluctuation in set 2 controls, unequivocally demonstrates that *D. chlorelloides* resistant cells arose by rare spontaneous mutations and not through direct and specific adaptation in response to VPW. However, it should be noted that it would be difficult to observe post-selective mutations (i.e. adaptive mutations) using fluctuation analysis if the rate of these kinds of mutations for VPW-resistance were $<10^{-8}$. Despite this, the rapid lethal effect of VPW seems unlikely to allow the appearance of adaptive mutations, because these kinds of mutations occur in non-proliferating microbial populations after being incubated on non-lethal selective medium (Foster 2000).

The mutation rate of VPW-sensitivity to VPW-resistance transformation in *D. chlorelloides* (4.7×10^{-7} mutants per cell per generation) was comparable to that from sensitivity to resistance to the sulfureous water from La Hedionda spa (southern Spain) in the chlorophycean *Spirogyra insignis* (Hassall) Kützing (2.7×10^{-7} mutants per cell per generation; Flores-Moya *et al.* 2005). These mutation rates were one or two orders of magnitude lower than those we have described for resistance to other biocides in cyanobacteria and micro-algae (Costas *et al.* 2001; López-Rodas *et al.* 2001, 2007, 2008a,b; Baos *et al.* 2002; García-Villada *et al.* 2002, 2004; Costas *et al.* 2007). Taking into account the countless cells comprising algal populations and the magnitude of the mutation rate allowing adaptation to VPW found in *D. chlorelloides*, it could be hypothesized that algae colonized the sulfureous, acid, warm, shallow pond from Vulcano Island rapidly after the last eruption (1888 to 1890). Our results suggest that the eukaryotic algae living in the hostile ecosystem of the Vulcano Island pond could be the descendents of chance resistant mutants that fortuitously arrived from 1890 onwards. Moreover, this colonization could be happening continuously since the last eruption and possibly occurred also in the past, in the ponds that disappeared with the last eruption. A recent similar hypothesis (continuous arrival of mesophilic micro-organisms) has been proposed to explain the pioneer bacteria communities inhabiting volcanic areas, where extremophilic strains were not detected (Portillo & González 2008). However, in view of the fact that few lucky resistant mutants of freshwater algae can arrive at Vulcano Island because of its isolation from other freshwater habitats, the phytoplankton species richness of this sulfureous, acid, warm, shallow pond of Vulcano Island remains limited.

The rapid adaptation of *D. chlorelloides* to the geothermal waters from Vulcano Island could be a model for understanding the survival of photosynthesizers during one of the most critical periods in the history of life: the Neoproterozoic 'snowball Earth'. The 'snowball

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Earth' hypothesis proposes that a series of global glaciations, reaching the Equator, occurred 850–750 million years ago (Kirschvink 1992). Negative carbon isotope anomalies in carbonate rocks from this period have been interpreted as the consequence of the collapse of primary production in the surface ocean for millions of years (Hoffman *et al.* 1998; Rothman *et al.* 2003), although some photosynthesizers could have survived in refugia associated with volcanic areas, such as hot springs or geothermal ponds (Schrag & Hoffman 2001). But some such refugia, which may have lethal concentrations of dissolved substances such as sulfur compounds and heavy metals, and acidic or very acidic pH, are often lethal for cyanobacteria and algae, which were the only photosynthesizers present in the Proterozoic biosphere (Kaufman *et al.* 1997). In spite of this, the selection of rare, spontaneous mutants could be enough to assure the adaptation of algae to stressful, hostile geothermal waters.

Cyanobacteria were not detected in the Vulcano Island pond. In fact, these organisms have been reported to be absent from moderately or extremely acidic environments (Brock 1973; Knoll & Bauld 1989; Albertano 1995; reviewed by Gimmler 2001), and a pH limit of 4.8 was postulated for proliferation of cyanobacteria (Brock 1973). However, the presence of filamentous cyanobacteria in acid lakes (pH 2.9) in Germany (Steinberg *et al.* 1998) renders doubtful this postulated lower pH limit. The presence of cyanobacteria in acidic environments may be very unusual, and our results are in agreement with this notion. In previous fluctuation experiments with cyanobacteria, using as selective agent acid waters from Spain's Rio Tinto (southwest Spain) (Costas *et al.* 2007), adaptation was not detected. Thus, neither selection on spontaneous mutations that occur before exposure to acidic water, nor specific adaptation during the exposure to acidic water, was enough to allow adaptation of the cyanobacterium *M. aeruginosa*.

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I.III. Genetic adaptation and acclimation of phytoplankters along a stress gradient in the extreme waters of the Agrio River Caviahue system (Neuquén, Argentina)

Referencia:

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CAPITULO II

ESTRATEGIAS GENÉTICAS DE LA ADAPTACIÓN RÁPIDA A CONTAMINANTES ANTROPOGÉNICOS

"Un error que cometemos es creer que somos los reyes de la Tierra y que tenemos derecho a decidir el futuro. Y como tenemos poder para hacerlo, no nos preocupamos por los problemas que causamos a otros organismos, otras especies o el entorno.".

(Stephen Jay Gould. Paleontólogo)

Uno de los factores desencadenantes del cambio ambiental global es la liberación al medio de sustancias tóxicas derivadas de la actividad humana. El impacto de la actividad antropogénica está dando lugar a una aceleración del cambio evolutivo en las distintas especies de organismos debido a la fuerte presión de selección ejercida (Palumbi 2001). El uso masivo de antibióticos, metales pesados, herbicidas, pesticidas, así como toda una serie de compuestos de nueva síntesis provoca la liberación de residuos al medio natural donde los organismos tienen que luchar por asegurar su supervivencia, y con ella, la de su especie. La capacidad de supervivencia dependerá del tipo e intensidad de la presión selectiva así como la capacidad intrínseca del organismo para el desarrollo de la resistencia en último término.

En el presente capítulo se estudia la capacidad adaptativa de distintas especies fitoplanctónicas de agua dulce pertenecientes a la división Chlorophyta: *Dyctiosphaerium chlorelloides y Scenenedesmus intermedius*, frente a distintos contaminantes de origen antropogénico. Como herramienta experimental se utiliza el Análisis de Fluctuación modificado para cultivos líquidos mediante el cual se puede determinar la capacidad de adaptación y la naturaleza de la mutación que confiere la

resistencia ante un agente selectivo concreto. Entre los contaminantes antropogénicos se han seleccionado cinco de distinta naturaleza que se han agrupado en 4 trabajos distintos para su estudio:

- En el primer trabajo se estudia la adaptación al Formaldehido. Esta sustancia química ha sido ampliamente utilizada como desinfectante, fumigante, así como reactivo analítico (EPA 1988; WHO 1989). Debido a su frecuente utilización así como su toxicidad aguda para el fitoplancton (Burridge *et al.* 1995) consideramos interesante el estudio de la capacidad de adaptación de los organismos frente a esta sustancia.
- En el segundo trabajo se seleccionó como agente selectivo el Cloranfenicol, un antibiótico de amplio espectro y comúnmente utilizado en la práctica veterinaria así como en la industria acuícola (Uriarte *et al.* 2001). El cloranfenicol se selecciona como representante para el estudio de la capacidad adaptativa de microalgas a un antibiótico.
- En el tercer trabajo se utiliza un metal pesado: Cromo (IV). El cromo se considera un contaminante acuático importante (Armienta-Hernández y Rodríguez-Castillo 1995) debido a la liberación de efluentes desde distintas industrias. Dentro de las distintas formas del cromo, el cromo IV se considera el más tóxico (Katz y Salem 1993) para las especies de microorganismos acuáticas.
- En el último trabajo se analiza la capacidad adaptativa de ambas especies fitoplanctónicas a dos herbicidas ampliamente utilizados: simazina y Diquat. Una de las causas principales de la crisis de la biodiversidad deriva del uso masivo de herbicidas en las actividades agrícolas con una fuerte actividad biocida (Tilman 1999). Por ello, el estudio de los mecanismos de adaptación del fitoplancton ante estos contaminantes residuales resulta de interés.

Los resultados de los cuatro trabajos muestran que las poblaciones microalgales de ambas especies son capaces de adaptarse a concentraciones letales de todas las sustancias examinadas gracias a la presencia de mutantes resistentes. En todos los casos, los mutantes aparecen gracias a mutaciones espontáneas que ocurren al azar, antes de que el organismo entre en contacto con la sustancia tóxica.

II.I. Adaptation of phytoplankton to novel residual materials of water pollution: an experimental model analyzing the evolution of an experimental microalgal population under formaldehyde contamination

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Adaptation of Phytoplankton to Novel Residual Materials of Water Pollution: An Experimental Model Analysing the Evolution of an Experimental Microalgal Population Under Formaldehyde Contamination

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Abstract The adaptation mechanisms of microalgae to grow in contaminated waters were analysed using a *chlorophyta* species under formaldehyde exposure as experimental model. Cultures initially collapsed after exposure to 16 ppm formaldehyde, but occasionally resistant cells were able to grow after further incubation. Resistant cells arose by rare spontaneous mutations that appeared before the exposure to formaldehyde (mutation rate = 3.61×10^{-6}), and not as result of physiological mechanisms. Although mutations may be the mechanisms that should allow the survival of microalgae in polluted waters in a world under rapid global change, mutants have a diminished growth rate.

Keywords Adaptation · Formaldehyde · Mutation · Water pollution

Water pollution by anthropogenic substances is a problem of great magnitude that urgently needs more basic research to facilitate predictions about the future and to determine actions to mitigate this environmental crisis. In this sense, studies focused on knowing if essential microbes succumb to anthropogenic toxins are of great importance. Particularly, the tolerance of microalgae to contaminated environments is very relevant from an ecological point of view, as these organisms are the principal primary producers of aquatic ecosystems.

Among these toxics, formaldehyde has become widely used as a chemical intermediate, analytical reagent, in concrete and plaster additives, wood preservation, in agriculture, disinfectants and fumigants (EPA 1988; WHO 1989). Formaldehyde has a half-life of 24–168 h in surface waters and 48–336 h in deeper waters (Howards et al. 1991), causing acute toxicity in phytoplankton (Chiayvareesajja and Boyd 1993; Burridge et al. 1995).

In order to study adaptation of microalgae to grow and survive in formaldehyde-polluted environments, a fluctuation analysis (Luria and Delbrük 1943) was performed. Usually, formaldehyde treatment produces massive destruction of microalgae (Chiayvareesajja and Boyd 1993; Burridge et al. 1995), but some cell variants could survive in formaldehyde-contaminated environments. The fluctuation test (Luria and Delbrück 1943) provides the appropriate procedure to discriminate between adaptation by selection of rare spontaneous mutations and other procedures. Recently, fluctuation test has been conducted entirely in liquid media, growing microalgae cultures first in a benign medium and then exposing them to contaminants (López-Rodas et al. 2001, 2007; Costas et al. 2001, 2007; Baos et al. 2002; García-Villada et al. 2002; Flores-Moya et al. 2005).

Materials and Methods

Experiments were performed with a wild-type strain of *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman (Chlorophyta) isolated from a pristine lagoon (without previous formaldehyde contamination) in Sierra

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Nevada (SE Spain). This strain was isolated from a single cell to assure no genetic variability within it. Before the experiments, cells were grown axenically in cell-culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany) at 22°C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Cultures were axenically maintained in mid-log exponential growth by serial transfers of subcultures to fresh medium.

To determine formaldehyde toxicity, the effects of increasing doses of formaldehyde on growth and photosynthetic performance of *Dictyosphaerium chlorelloides* were measured. Experimental cultures were seeded each with 1.3×10^6 cells from mid-log exponentially growing cultures. A stock solution of about 38% formaldehyde (Sigma, Aldrich Chemie, Taufkirchen, Germany) was prepared in BG-11 medium to obtain serial dilutions of 1.60×10^{-3} % w/w (16 μg/mL), 9.94×10^{-4} % w/w (10.6 μg/mL), 6.14×10^{-4} % w/w (6.4 μg/mL), and 3.80×10^{-4} % w/w (4 μg/mL) to be used for algal exposure. Three replicate cultures of each formaldehyde concentration as well as three unexposed controls were prepared. In these cultures and controls, growth rate (m) was calculated using the equation:

$$m = \frac{Log_e \frac{N_t}{N_0}}{t}, \quad \text{(Crow and Kimura 1970)}$$

where $t = 7$ d, and N_0 and N_t are the cell numbers at the start and at the end of the experiment, respectively. Experiments and controls were counted using a spectrofluorimeter (Schimadzu RF-551S, Durisburg, Germany) relating the chlorophyll a fluorescence with cell density within the lineal range.

The effective quantum yield (Φ_{PSII}) was also measured in experiments and controls using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) 24 h after formaldehyde exposure. Effective quantum yield was calculated as follows:

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m}$$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al. 1986).

The fluctuation analysis (Fig. 1) was performed at 22°C and under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, and consisted of two Sets of culture flasks. Set 1 included 105 parallel cell-culture flasks, each one initially seeded with $N_0 = 125$ cells (i.e. a small number enough to assure the absence of pre-existing mutants). These cultures were allowed to grow (as previously detailed) until they reached approximately $N_t = 4.2 \times 10^5$ cells per flask, and then BG-11 medium containing formaldehyde (final concentration 1.6×10^{-3} (16 μg/ml) % w/w) was added. Control (Set 2) consisted on 25 parallel cell-culture flasks containing each 4.2×10^5 cells from the same parental population and with the same concentration of formaldehyde in BG-11 medium as Set 1. Both Sets were inoculated simultaneously. Cultures were grown for 50 days and then resistant cells in each culture were detected using a spectrofluorimeter (Schimadzu RF-551S, Durisburg, Germany). If resistant cells arose only from spontaneous mutations before selection (formaldehyde addition), then a high variance in their presence per culture (fluctuation) should be found as the chance of mutation would occur earlier in some cultures, later or even not occur in others. On the opposite, if resistant cells arose only in response to the selective medium, physiological mechanisms or post-adaptive mutation, every cell should present the same (and low) probability to adapt to the new medium. Thus, their distribution per culture should not exhibit any fluctuation at all. The control (Set 2) estimates the error in sampling resistant cells. Since this Set 2 is the experimental control of the analysis of fluctuation, a similar variance/mean ratio between Sets 1 and 2, would confirm that resistant cells arose in response to the selective medium.

The mutation rate from formaldehyde sensitive to formaldehyde-resistant cells was estimated by fluctuation analysis. The proportion of cultures from Set 1 showing no resistant cells after formaldehyde exposure was the parameter (P_0 estimator) used to calculate the mutation rate (μ). The P_0 estimator (Luria and Delbrück 1943) is defined as follows:

$$P_0 = e^{-\mu(N_t - N_0)}$$

where P_0 is the proportion of cultures showing no resistant cells, and N_0 and N_t are the initial and the final population size, respectively.

Therefore, μ (mutation rate) was calculated as:

$$\mu = \frac{-\mathrm{Log}_e P_0}{N_t - N_0}$$

The mutation from a normal wild-type formaldehyde-sensitive allele to a formaldehyde-resistant allele is recurrent. In addition, the formaldehyde-resistant allele is detrimental to fitness in the absence of formaldehyde. As a result, new resistant mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance (Crow and Kimura 1970). At each time there will be a certain number of resistant cells that are not yet eliminated. The average number of such mutants will be determined by the balance between mutation rate and selective elimination rate, in accordance with the equation:

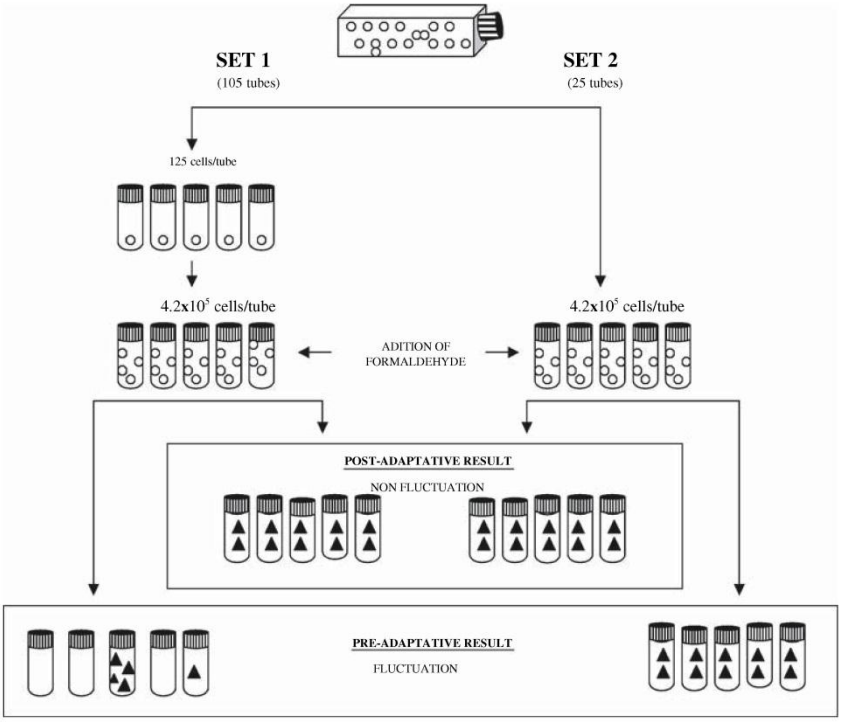

Fig. 1 Schematic diagram of the experiment modified from the classic Luria and Delbrück(1943) fluctuation analysis. Set 1 consists on 105 cultures each one containing 125 cells. They were allowed to grow, before adding the substance in study, till they reached the number of 4.2×10^5 cells. Set 2 consists on 25 tubes control with 4.2×10^5 cells that directly incorporated the studied product. If the adaptation to medium is due to the uncommon pre-selective mutations, between both Sets the existence of a huge fluctuation should be evident, as mutation appears by chance. In Set 1, some tubes would contain some mutants that had appeared early during cell division, in other tubes mutants would have appeared later, and, in the rest of them, there would be no mutants at all. On the other hand, if the resistance needs specific adaptation in response to formaldehyde exposure, both Sets 1 and 2, would be very similar, as every cell would have the same small probability to survive in that medium

$$q = \sqrt{\frac{\mu}{s+\mu}} \quad \text{(Kimura and Maruyama 1966)}$$

where q is the frequency of the formaldehyde-resistant allele, μ is the mutation rate and s is the coefficient of selection calculated as follows:

$$s = 1 - \frac{m_f^r}{m_f^s}$$

where m_f^r and m_f^s are the fitness of formaldehyde-resistant and formaldehyde-sensitive cells, respectively (Crow and Kimura 1970).

Results and Discussion

Low concentrations of formaldehyde have significant toxic effects on wild-type *D. chlorelloides* cells (Table 1). Growth rate and photosynthesis performance were severely reduced even by 6.4 μg/mL, whereas concentrations of 16 μg/mL inhibited completely growth and photosynthesis performance.

When microalgae were treated with 16 μg/mL formaldehyde in Set 1, all cultures initially collapsed due to the destruction of sensitive cells by the toxicant. But some cells were able to grow in some culture flask after 50 days, suggesting that rare formaldehyde-resistant cells occur (Table 2). A high fluctuation in the number of resistant cells per culture was observed in Set 1 (from 0 to more than 2.6×10^8 resistant cells per culture flasks). In contrast in Set 2 all the cell cultures contain formaldehyde-resistant cells showing low fluctuation (Table 2).

The mutation rate from formaldehyde susceptibility to formaldehyde resistance in *D. chlorelloides* (3.61×10^{-6} divisions) was found to be on the same order of magnitude than mutation rates we have described for resistance to many other biocides in chlorophyta (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al. 2002), significantly higher than mutation rates for

Table 1 Inhibition of growth and photosynthetic performance (effective quantum yield) of *Dictyosphaerium chlorelloides* by increasing doses of formaldehyde, calculated as percentage of untreated controls (dose-effect)

Formaldehyde concentration yield (μg mL^{-1}) (mean ± SE)	Growth rate inhibition (%) (mean ± SE)	Effective quantum inhibition (%)
0.0	0 ± 0	0 ± 0
4.0	17 ± 6	5 ± 0
6.4	61 ± 5	13 ± 1
10.6	100 ± 0	98 ± 2
16.0	100 ± 0	100 ± 0

Table 2 Fluctuation analysis of *Dictyosphaerium chlorelloides* exposed to formaldehyde (16 μg/mL)

	Set 1	Set 2
No. of culture replicates	105	25
N_0 (cells)	125	–
N_t (cells)	4.2×10^5	4.2×10^5
No. of cultures containing the following no. of formaldehyde-resistant cells:		
0	23	0
$1–10^7$	32	0
$10^7–2 \times 10^7$	11	0
$2 \times 10^7–3 \times 10^7$	8	0
$>3 \times 10^7$	31	25
Mutation rate (mutants per cell division) 3.61×10^{-6}		

sulphurous water of La Hedionda, 2.7×10^{-7} (Flores-Moya et al. 2005) and on the same order of magnitude than mutation rates for sulphurous water of Spain's Tinto River (Costas et al. 2007). Some stressful environments support populations of algal species at the extreme limits of their physiological tolerance (Fogg 2001). Algae survive in such hostile environments as a result of physiological acclimation by modifications of gene expression (Belfiore and Anderson 2001). Beyond physiological limits, adaptive evolution depends on the occurrence of new mutations that confer resistance (Belfiore and Anderson 2001).

On the opposite of formaldehyde-sensitive wild-type algae, the formaldehyde-resistant mutants isolated from Set 1 were able to grow under 16 μg/mL of formaldehyde. Furthermore, such a high formaldehyde concentration just inhibited 75% of their quantum yield. Isolated formaldehyde-resistant mutants growing in absence of formaldehyde showed a coefficient of selection of $s = 0.06$ respect to the wild-type formaldehyde-sensitive cells. Since mutation is recurrent, but mutant is usually detrimental in fitness, in each generation new mutants arise, but most of them are finally eliminated by natural selection (Crow and Kimura 1970). The frequency (q) of formaldehyde-resistant alleles in non-extreme environment was estimated, by using the values of μ and s, in 7.68 formaldehyde-resistant mutants per 10^3 cells, as the consequence of the balance between mutation and selection. Consequently, the ancestral microalgae population would be predominantly constituted by a clone line of wild-type sensitive genotype and, simultaneously, in a very small fraction, by a clone line of formaldehyde-resistant mutants. Thus, a rare spontaneous mutation from formaldehyde susceptibility to formaldehyde resistance seems to be enough to assure survival of microalgae populations in formaldehyde-contaminated environments.

Synthetic chemicals, like formaldehyde, causing water pollution could exert drastic selective pressures to facilitate rapid fixation of rare pre-adaptive mutations in natural populations of microalgae. Although some phytoplankton species could be able to rapidly adapt to new residual substances, such process usually implies a high cost for the ecosystem, as they reduce growth and photosynthetic performances.

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II.II. Toxic effects and adaptation in Scenedesmus intermedius to anthropogenic chloramphenicol contamination: genetic versus physiological mechanisms to rapid acquisition of xenobiotic resistance

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Toxic effect and adaptation in *Scenedesmus intermedius* to anthropogenic chloramphenicol contamination: genetic versus physiological mechanisms to rapid acquisition of xenobiotic resistance

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Abstract Anthropogenic water pollution is producing a challenge to the survival of phytoplankton populations. From an ecological point of view, the tolerance of these microorganisms to water pollution is of paramount importance since they are the principal primary producers of aquatic ecosystems. The adaptation of a common chlorophyta species (*Scenedesmus intermedius*) exposed to selected dose-response chloramphenicol (CAP) concentrations has been analyzed. A fluctuation analysis demonstrated that CAP-resistant cells arise due to spontaneous mutation which occurs randomly prior to the antibiotic exposure. CAP-inhibited growth and photosynthetic performance of algal cells at 0.28 mg/l, and the $IC_{50(72)}$ value was established in 0.10 mg/l for both parameters. The mutation rate from CAP sensitivity to resistance was 1.01×10^{-5} mutations per cell division, while the frequency of CAP-resistant allele in non-polluted environment was estimated to be 5.5 CAP-resistant mutants per 10^3 sensitive-cells. These results demonstrate that resistant mutants exhibit a diminished fitness until 5 mg/l of CAP, thus enabling the survival of microalgae population.

Keywords Chloramphenicol · *Scenedesmus intermedius* · Toxicity · Mutation rate · Fluctuation analysis

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Introduction

Human activities are changing biosphere-level processes (global change) and causing biodiversity crisis (Woodruff 2001; Myers and Knoll 2001). New substances are polluting water and causing environmental catastrophes in inland water systems. This is a problem of the utmost importance with basic research urgently needed to provide useful information to make future predictions, so that strategies may be designed to mitigate this environmental crisis (Ehrlich 2001). To this end, studies focused on discovering if essential microbes succumb to anthropogenic toxins are very significant (Woodruff 2001) since human activities are the greatest evolutionary force (Palumbi 2001). As microalgae are the primary producers of aquatic ecosystems (Kirk 1994; Falkowski and Raven 1997), the tolerance of these microorganisms to contaminated environments is very important from an ecological point of view.

Antibiotics play a major role in modern agriculture and aquaculture activities with their use increasing in many developed nations (Sarmah et al. 2006). These drugs are mainly administered through medicated feed. This practice may result in the antibiotics entering the environment by leaching from uneaten feeds, unabsorbed particles present in manure, or from the discharge of aquatic animals (Robinson et al. 2007). Studies performed in intensive fish farms have demonstrated that about 70–80% of applied antibiotics administered to fish as food additives end up in the aquatic environment. This may result in adverse ecological effects, including the development of resistant bacterial populations, direct toxicity to microbiota, and/or possible risks in the transfer of these antibiotic resistances to human pathogenic microbes (Rigos et al. 2004). Therefore, the overuse and misuse of certain antibiotics has

led to several adverse environmental effects and raised public concern (Sarmah et al. 2006).

Chloramphenicol (CAP) is a broad-spectrum antibiotic, commonly used in veterinary and aquaculture practice as a chemotherapeutic agent to control diseases (Uriarte et al. 2001). It prevents the synthesis of proteins by binding onto ribosomes in the same location where mRNA binds to ribosomes. Through this process, CAP also halts the synthesis of proteins in chloroplasts (Pogo and Pogo 1965; Anderson and Smillie 1966).

The main goals of this work were determine the toxic effect of CAP on microalgae, to establish the capacity of microalgae to survive in CAP contaminated environments as the result of rapid adaptation, and to verify if rapid adaptation to high doses of CAP is due to a process of physiological acclimation or genetic adaptation. CAP-resistance is a very complex problem, with important environmental and health implications (Levy 2002).

Within limits, organisms may survive in chemically contaminated environments as a result of two different processes: physiological acclimation which usually results from modifications of gene expression, and genetic adaptation by natural selection due to the occurrence of mutations which provide the appropriate resistance (Belfiore and Anderson 2001). Usually, the work of ecotoxicologists is focused toward physiological level. In contrast, rapid genetic adaptation to contaminated environments has been scarcely studied.

The statistical analysis was performed using a Luria-Delbrück Fluctuation Analysis (1943) modified as previously described (López-Rodas et al. 2001; Costas et al. 2001) which allows for differences between physiological acclimation and genetic adaptation. In previous works focused on understanding the mechanisms of microalgae adaptation to extreme natural environments (Lopez-Rodas et al. 2008a, b; Costas et al. 2007, 2008; Flores-Moya et al. 2005), fluctuation analysis proved to be useful to differentiate between these two mechanisms.

Materials and methods

Experimental organisms and culture conditions

The experiments where performed with chloroficeae *Scenedesmus intermedius* isolated from a pristine lagoon in Doñana National Park (SW Spain). This is a stock strain stored in the algal culture collection of the Complutense University (Madrid, Spain). *S. intermedius* was grown axenically in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma Aldrich Chemie, Taufkirchen, Germany), at 20°C under continuous light of 60 μmol m^{-2} s^{-1} over a waveband of 400–700 nm. Cultures were maintained at mid-log exponential growth by serial transfers of cell inoculums to fresh medium. Prior to beginning the experiments, the cultures were re-cloned by isolating a single cell to avoid including any previous spontaneous mutants accumulated in the cultures.

Effect of CAP on the growth rate and photosynthetic performance

Chloramphenicol (CAP, ≥ 98% purity from Sigma-Aldrich Chemical Co. St. Louis, MO, USA) was used dissolved in distilled water. The toxic effect of CAP on the growth rate of *S. intermedius* was initially tested using 13 ml polystyrene sterile tubes (Sarstedt Co., Nümbrecht, Germany). No adherence to the tube walls of either chemicals or microalgae was previously checked. Neither chemicals nor microalgae were observed to adhere to the tube walls. Serial dilutions 0.05, 0.1, 0.15, and 0.2 mg/l of CAP were prepared in BG-11 medium. Eight replicates were inoculated with 10^4 cells from mid-log exponentially growing cell cultures for each concentration.

The effect of CAP was estimated after 72 h by calculating the acclimated maximal growth rate (m) in mid-log exponentially growing cells, that derives from the equation: $N_t = N_0 e^{mt}$, where $t = 3$ days, N_t are the cell numbers at the end of the experiment and N_0 are the cell numbers at the beginning of the experiment. Therefore, m was calculated as: $m = \log_e (N_t/N_0)/t$. Acclimated maximal growth rate (m) is the Malthusian parameter of fitness under conditions of r selection (Crow and Kimura 1970; Spiess 1989). Experiments and controls were blind trials (i.e., the person counting the test did not know the identity of the tested samples) done/repeated every 24 h using a haemocytometer in an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany). The number of samples in each case was determined by using the progressive mean procedure (Williams 1977) which assures a counting error below 5%.

The photosynthetic response was measured as an effective fluorescence quantum yield (Φ_{PSII}) with experiments and controls done in triplicate using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) at 72 h. Effective quantum yields were calculated as follows: $\Phi_{PSII} = (F'_m - F_t)/F'_m$, where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al. 1986).

A concentration causing 50% growth inhibition in algae was evaluated according to the 'area under the curve' method prescribed by the ISO (1982). Seventy-two hour mean and 100% inhibitory concentration values ($IC_{50(72)}$ and $IC_{100(72)}$, respectively) were determined by nonlinear regression analysis with all results expressed as mean ± SD. Data were presented as an inhibition percentage of growth rate and Φ_{PSII} with regard to control Statistical analysis was

performed using the computer software package GraphPad Prism v 4.0 (Graph-Pad Software Inc, USA).

Fluctuation analysis: from CAP-sensitive to CAP-resistant cells

Scenedesmus intermedius could conceivably adapt to lethal doses of CAP through a selection process of rare spontaneous mutations occurring prior to CAP exposure. Furthermore, this could transpire due to a direct and specific response to the CAP, specifically a physiological acclimation or post-selective mutations that occur as a result of CAP. Fluctuation analysis allows distinguishing between CAP-resistant cells which originated from random spontaneous mutations occurring prior to CAP exposure, and CAP-resistant cells arising through adaptation acquired during the exposure to CAP.

A fluctuation analysis consists of experiments (set 1) and its controls (set 2). In the first set of experiments 100 tubes were inoculated with a sufficiently small number of wild-type cells to ensure that no pre-existing mutants were present (in this experiment $N_0 = 10^2$ cells per tube). In the beginning of the experiment, the cultures were grown without CAP. As the cells divide, some may randomly mutate from CAP-sensitivity to CAP-resistance which gives rise to mutant clones. At the end of the growing period cultures grew until $N_t = 1.13 \times 10^5$ cells. At this point, cultures were supplemented with fresh liquid medium containing 5 mg/l of CAP (the selective agent). Such a dose corresponds approximately to 20 times the $IC_{100(72)}$, value obtained in previous acute toxicity assays (and inhibits the growth of the wild-type CAP-sensitive algae). Only CAP-resistant mutants would be able to grow under a load of 5 mg/l of CAP. Tubes were periodically checked to detect CAP-resistant cells. At the end of 2 months of culturing using CAP, which was adequate time to ensure that resistant mutant cells could generate enough progeny to be detected, all the tubes were counted.

Chloramphenicol (CAP)-resistant cells could be explained in two ways: if resistant cells arise through a direct and specific response to CAP, each cell would have a similar probability of survival and the number of CAP-resistant cells would be similar in all tubes. If so, inter-tube variation would be consistent with the Poisson model (i.e., the variance of these replicate samples would be equal to the mean). In contrast, if CAP-resistant cells arise through rare spontaneous mutations that occur prior to CAP exposure, in some cultures mutation may occur early and many of the progeny would be resistant. In other cultures, mutation could appear during a later cell division resulting in only a few resistant progeny. In still others, no mutation may occur so that all of the progeny would be sensitive. Therefore, inter-tube variation would not be consistent with the Poisson model (i.e., the variance of these replicate samples would be higher than the mean).

In set 2 (control), 25 aliquots of 1.13×10^5 cells from the same parental population were separately transferred to culture flasks containing fresh liquid medium containing 5 mg/l CAP. The number of cells in each tube was similar since variation is due only to random sampling and variation from tube to tube would be consistent with the Poisson model. Since set 2 constitutes the experimental control for the fluctuation analysis, if a similar variance/mean ratio between set 1 and set 2 is found, resistant cells would appear as a result of a direct and specific response to CAP. This could be a physiological acclimation or due to post-selective mutations that occur as a result of CAP. In contrast, if the variance/mean ratio in set 1 is higher than the variance/mean ratio in set 2 (fluctuation), this will confirm that resistant cells appear by through a rare spontaneous mutation.

In addition, the fluctuation analysis allows estimating the mutation rate (μ) by means of the P_0 estimator which represents the proportion of cultures showing no mutant colonies after CAP exposure in the first set of experiments. The P_0 estimator (Luria and Delbrück 1943) was calculated as follows:

$$P_0 = e^{-\mu(N_t - N_0)}$$

where P_0 is the proportion of cultures showing no resistant cells, N_0 is the initial cell population size, and N_t is the final cell population size. Thus, μ was calculated as (Luria and Delbrück 1943):

$$\mu = -\mathrm{Log}P_0/(N_t - N_0).$$

Characterization of CAP-resistant mutants

Chloramphenicol (CAP)-resistant cells were randomly isolated from set 1 cultures and grown to mass populations. Fitness CAP-resistant mutants and CAP-sensitive wild-type cells were characterized under conditions of selection in culture medium without CAP, as presented in a concentration-effect study. Three replicates of CAP-resistant mutants and three controls (CAP-sensitive wild-type cells) were grown in culture medium. After 4 days, cell numbers in experimental and control groups were counted using an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany).

Mutation-selection equilibrium

The mutation from CAP-sensitive wild-type allele to a CAP-resistant allele is recurrent. Furthermore, CAP-resistant allele is detrimental in fitness in the absence of the antibiotic. Luria and Delbrück fluctuation analysis demonstrated that new resistant mutations arise in each

generation, but most of these strains are eliminated eventually by natural selection or by chance (Crow and Kimura 1970; Spiess 1989). There is equilibrium among resistant mutants that arise by mutation, and the ones that disappear in a population in non-selective conditions. The average number of such mutants will be determined by a balance between the mutation rate and the rate of selective elimination. This is in accordance with the equation $q = (\mu/s)^{1/2}$ (Kimura and Maruyama 1966) where q is the frequency of the CAP-resistant allele and s is the coefficient of selection calculated as $s = 1 - (m_C^r/m_C^s)$ where m_C^r is the Malthusian fitness of CAP-resistant cells and m_C^s is the Malthusian fitness of CAP-sensitive cells, both of which are measured under non-selective conditions.

Results

Effect of CAP on growth rate and photosynthetic performance

Chloramphenicol (CAP) had acute toxicity on sensitive microalgae, inhibiting both cell growth and photosynthetic performance (Table 1). The $IC_{50(72)}$ values obtained for growth inhibition were similar to those obtained for $\Phi_{(PSII)}$ quantum yield assays. No statistical differences between growth rate and $\Phi_{(PSII)}$ quantum yield assays were found when comparing linear regression analysis for both parameters (Fig. 1).

Fluctuation analysis

When an analysis of fluctuation was carried out exposing wild-type cells to an inhibitory concentration of CAP (5 mg/l), cell density was reduced in set 1 and set 2 cultures due to the effect of the antibiotic. However, after further incubation for 60 days, several of the cultures were able to re-establish growth capacity demonstrating that CAP-resistant cells do occur. A high fluctuation in the number of resistant cells per culture was observed in set 1 ranging from 0 to more than 10^7 resistant-cells per culture flask. In set 2, by contrast, all cell cultures contained CAP-resistant cells in the order of magnitude of 10^7 indicating low fluctuation (Table 2). Moreover, the variance/mean ratio in set 1 was far higher than the variance/mean ratio in set 2 ($CV_{set1}/CV_{set2} = 13.26$). From this information it can be inferred that CAP-resistant cells arose as a result of a rare spontaneous mutation that occur randomly prior to CAP exposure.

The mutation rate (μ) estimated from sensitivity to resistance for CAP in *S. intermedius* was 1.01×10^{-5} mutants per cell division (Table 2).

Table 1 Comparison of 72-h IC_{50} and IC_{100} values with their associated 95% confidence limits (CL), expressed in mg/l, correspondent to growth inhibition and Φ_{PSII} inhibition assays in *Scenedesmus intermedius* cells exposed to chloramphenicol (CAP)

Parameter	*n*	[CAP] (mg/l)	
		Growth inhibition	Φ_{PSII} inhibition
$IC_{50(72)}$ (CL 95%)	8	0.10 (0.09–0.12)	0.10 (0.08–0.12)
$IC_{100(72)}$ (CL 95%)	8	0.25 (0.20–0.31)	0.28 (0.21–0.38)

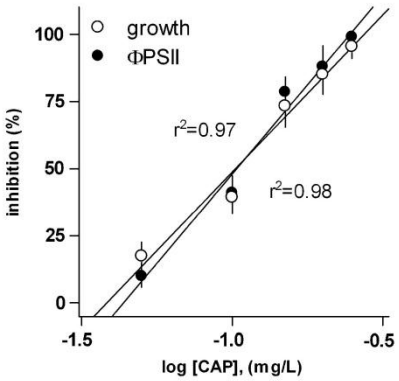

Fig. 1 Dose response relationship of chloramphenicol (CAP) to *Scenedesmus intermedius* in growth (○) and Φ_{PSII} (●) inhibition. *Points* represent means with *vertical lines* showing SD mean ($n = 8$)

Characterization of the CAP-resistant mutant

Chloramphenicol (CAP)-resistant mutants growing in BG-11 medium without CAP showed less fitness than the wild-type CAP-sensitive strains. By each doubling of the wild-type sensitive strains, CAP-resistant mutants were able only of 0.67 doublings. These relative values of fitness were used to compute the coefficient of selection (s) of

Table 2 Fluctuation analysis of wild-type *Scenedesmus intermedius* exposed to a growth and photosynthetic chloramphenicol-inhibitory concentration (5 mg/l)

	Set 1	Set 2
N_0 of culture replicates	100	25
N_0 (N^o cells)	100	–
N_t	3.1×10^5	
N_0 of cultures containing the following no. of chloramphenicol resistant cells		
0	4	0
10^5–10^6	3	0
10^6–10^7	16	0
10^7–10^8	77	25
CV_{set1}/CV_{set2}	13.26	
Mutation rate	1.01×10^{-5}	

CAP-resistant mutants ($s = 0.33$). By using previous μ and s values, the frequency (q) of resistant alleles as a consequence of the balance between mutation and selection was calculated. A frequency (q) of 5.5 CAP-resistant mutants per 10^3 sensitive-cells could be maintained in the absence of CAP as the result of the equilibrium between recurrent mutation and selection.

Discussion

The most commonly used antibiotics in aquaculture are: thiamphenicol, CAP, streptomycin, florfenicol, kanamycin, oxytetracycline, neomycin, and oxolinic acid. Previous studies using test systems have shown that various antibiotics remain active in wastewaters against different groups of bacteria, microflora and microfauna (Christensen et al. 2006; Quinn et al. 2008). In addition, growth inhibition effects of several antibiotics against algae and daphnids have been reported at concentrations of 5–100 mg/l (Holten Lützhoft et al. 1999; Wollenberger et al. 2000). Other authors have also recounted that several antibiotics used in intensive fish farming have various growth inhibition (EC_{50} 0.0037–3,108 mg/l) effects on algae, depending on the antibiotics and algae used (Halling-Sorensen 2000).

Results have shown that growth of *S. intermedius* is inhibited by CAP exposures. Though treatments with CAP in animal cultures are strictly prohibited, illegal utilizations are still being reported (Takino et al. 2003; Huang et al. 2006). If this antibiotic was to be used in larval culture systems (Gaunt et al. 2006; Uriarte et al. 2001), the feeding and growth of larval organisms could be adversely affected owing to the inhibition of algal populations by CAP. Furthermore, if released with effluent or overflow, CAP could potentially upset an aquatic environment. The residues of CAP from animal culture and/or human medication may result in the contamination of receiving waters.

For decades, fluctuation analysis has been considered the appropriate procedure to discriminate between resistant cells which have arisen by spontaneous mutation occurring randomly during replication of organisms prior to the exposure to a selective agent and resistant cells which arose from a direct and specific adaptation to a selective agent (i.e. CAP) (Cairns et al. 1988; Tlsty et al. 1989; Dijkmans et al. 1994). Fluctuation analysis has also been used to analyse mechanisms of algal resistance (García-Villada et al. 2002, 2004; Lopez-Rodas et al. 2007).

The large variation (fluctuation) in CAP-resistant cells in the set 1 experiment is in contrast with the minor variation in set 2 controls which unequivocally demonstrates that CAP resistance develops through a rare spontaneous mutation. This occurs prior to CAP exposure. Moreover, CAP did not stimulate the occurrence of CAP-resistant cells.

Recently, the molecular basis of CAP resistance has been revised (Siibak et al. 2009). Choramphenicol affects the assembly of both the large and the small subunits. New data suggests that CAP resistance in bacteria is due to the expression of a small resistance peptide acting on mature ribosomes (Siibak et al. 2009).

The rare mutation rate from CAP-sensitivity to CAP-resistance in *S. intermedius* (1.01×10^{-5} mutations per cell division) was found one order of magnitude higher than those previously described for the resistance to several biocides in microalgae such as formaldehyde (Lopez-Rodas et al. 2008c), erythromycin (López-Rodas et al. 2001), DCMU herbicide (Costas et al. 2001), TNT (García-Villada et al. 2002) or copper. The high mutation rate for CAP resistance may be consequence of the existence of several mechanisms of CAP resistance previously described such as reduced membrane permeability (Burns et al. 1985; Nikaido 1989), DNA ribosomal mutation (Blanc et al. 1981) and elaboration of CAP-acetyltransferase (Cohen et al. 1980). Subsequently, several mutations may confer resistance.

Chloramphenicol (CAP)-resistant cells to 5 mg/l exhibit diminished fitness in the absence of CAP compared to wild-type *S. intermedius* in the absence of CAP as the growth rate of CAP-resistant mutants is less than the wild-type CAP-sensitive cells. As a result, resistant cells are expected to be eliminated due to natural selection. However, in each generation new mutants arise creating a balance between mutation rate and selective elimination since mutation is recurrent. At the same time, there can be a certain number of mutant cells that will have not been eliminated yet. Thus, rare spontaneous mutation from CAP-sensitivity to CAP-resistance seems to be sufficient to assure the survival of microalgae populations in contaminated environments until 5 mg/l of CAP.

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II.III. Toxicity and adaptation of Dictyosphaerium chlorelloides to extreme chromium contamination

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TOXICITY AND ADAPTATION OF *DICTYOSPHAERIUM CHLORELLOIDES* TO EXTREME CHROMIUM CONTAMINATION

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Abstract—Metals are often spilled by industries into inland water environments, with adverse consequences. Numerous papers have reported that heavy metals produce massive destruction of algae. Nevertheless, algal populations seem to become tolerant when they have had previous exposures to heavy metals. Because the mechanisms allowing heavy metal tolerance of algae are not yet known, the present study analyzed the effect of hexavalent chromium on growth and photosynthetic performance of *Dictyosphaerium chlorelloides,* stressing on the adaptation mechanisms to chromium contamination. Growth and photosynthetic performance of algal cells were inhibited by Cr(VI) at 10 mg/L, and the 72-h median inhibition concentration was established as 1.64 and 1.54 mg/L, respectively. However, after further incubation for a three month period in an environment with 25 mg/L of chromium, some rare, chromium-resistant cells occasionally were found. A Luria–Delbrück fluctuation analysis was performed to distinguish between resistant algae arising from rare, spontaneous mutations and resistant algae arising from physiological adaptation and other adaptive mechanisms. Resistant cells arose only by spontaneous mutations before the addition of chromium, with a rate of 1.77×10^{-6} mutants per cell division. From a practical point of view, the use of both chromium-sensitive and chromium-resistant genotypes could make possible a specific algal biosensor for chromium.

Keywords—Adaptation Chromium *Dictyosphaerium chlorelloides* Mutation Toxicity

INTRODUCTION

Today, human activities are altering biosphere-level processes and causing a biodiversity crisis, with global extinction rates 500-fold above background [1,2]. As a result, several million geographically distinct populations and thousands of species go extinct annually [1]. Humans are the world's greatest evolutionary force, and great scientific effort should be made to comprehend the impact of civilization on the natural world [3,4].

The biodiversity crisis is reasonably well understood for terrestrial vertebrates and a few other groups, but little is known about this crisis in organisms as abundant and important as microorganisms. Studies with microorganisms would be interesting, however, because dependable nutrient cycles may become less predictable as essential microorganisms succumb to anthropogenic toxicants [1]. From an ecological point of view, the tolerance of these microorganisms to water pollution is paramount, because they are the majority primary producers of aquatic ecosystems [5]. Furthermore, from a physiological and genetic point of view, the survival and growth of microalgae in extremely contaminated environments is an interesting topic [6].

Heavy metals often are spilled from industrial sources into the environment. These metals are among the main man-made pollutants in aquatic ecosystems and a powerful anthropogenic selection force, with serious environmental implications and evolutionary consequences. Numerous papers have reported that heavy metals have a rapid effect on microalgae, causing massive destruction of sensitive cells [7,8]. These studies usually analyzed the injurious effects of heavy metals on microalgae and, indirectly, its effects on the aquatic ecosystems. Some heavy metal-resistant algal variants, however, may have succeeded by means of physiological or genetic mechanisms. The biological consequences of heavy metal contamination would be very different depending on the ability of microalgae to adapt to chromium. It seems that microalgal populations can be tolerant to the presence of heavy metals if they have had previous exposures [9,10].

In spite of this, little is known about the mechanisms allowing adaptation of microalgae to heavy metals. However, evidence for these mechanisms can be found in previous work that analyzed the effect of a toxic spill of acid wastes rich in heavy metals [11] or the effects of copper used as an algaecide in reservoirs [8,12]. Apparently, microalgae may survive exposure to heavy metals as a result of two different processes: First, physiological adjustment (acclimation or tolerance), usually resulting from modifications of gene expression [13], and second, genetic adaptation by natural selection if mutations provide the appropriate genetic variability, revealing the existence of resistant genotypes [14]. In this sense, a lot of interesting questions arise and require further work to be answered.

Chromium is widely used by industry and is an important water pollutant [15,16]. Untreated effluents from industrial discharges contain approximately 80 to 100 mg/L of Cr(VI)—much higher than the permissible limit of 0.05 to 1 mg/L [17]. The biological effects of chromium, which exists in hexavalent (VI) and trivalent (III) forms, depend on its oxidation state.

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The majority of Cr(VI) found in nature comes from industrial emissions, and Cr(VI) is the most toxic form of chromium to the plant, animal, and microorganism species that inhabit aquatic environments [18,19].

The present study has a double aim: First, to study the effect of heavy metals on the growth rate and photosynthetic performance of microalgae under a classic ecotoxicological approach, and second, to analyze the ability of sensitive cells to adapt to heavy metals under an evolutionary ecological procedure. The experimental model used Cr(VI) and *Dictyosphaerium chlorelloides* (Naumann, Komárek, and Perman), an abundant Chlorophyceae with a worldwide distribution that is widely used in studies of evolutionary ecology with algae.

MATERIALS AND METHODS

Experimental organism

Dictyosphaerium chlorelloides, strain Dc, was from the algal culture collection of the Genetics Laboratory, School of Veterinary Sciences, Complutense University. Cells grew axenically in culture flasks (Greiner; Bio-One), with 20 ml of BG-11 medium (Sigma Aldrich Chemie) at 20°C under a continuous light of 60 µmol/m²/s over the waveband of 400 to 700 nm. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculums to fresh medium once a fortnight. Before the experiments, the cultures were recloned (by isolating a single cell) to avoid including any previous spontaneous mutants that had accumulated in the cultures.

Effect of chromium on growth rate and photosynthetic performance

Hexavalent chromium oxide (Cr(VI); Sigma-Aldrich Chemical Co.) was used dissolved in distilled water. The toxic effect of Cr(VI) on the growth rate of *D. chlorelloides* was tested previously using 13-ml, polystyrene, sterile tubes (Sarstedt). Those previous studies determined the suitability of using polystyrene, sterile tubes for these toxicity assays, assuring that neither chemicals nor microalgal cells adhered to the tube walls [8,20]. Serial dilutions of Cr(VI) in BG-11 medium were prepared to obtain concentrations of 0, 0.1, 0.5, 1, 2, 5, and 10 mg/L. For each dose, eight replicates were inoculated with 10^4 cells/ml from mid-log, exponentially growing cell cultures.

The effect of Cr(VI) was estimated in both strains (sensitive and resistant to exposure) after 72 h by calculating the acclimated maximal growth rate (m) in mid-log, exponentially growing cells, which derives from the equation $N_t = N_0 e^{mt}$, where t is 3 d, N_t is the cell number at the end of the experiment, and N_0 is the cell number at the start of the experiment. Therefore, m was calculated as $m = \log_e(N_t/N_0)/t$. Acclimated maximal growth rate (m) is the Malthusian parameter of fitness under conditions of r selection [21,22]. Experiments and controls were performed under blind count (i.e., the person counting the test did not know the identity of the tested samples) every 24 h using a hemocytometer in an inverted microscope (Axiovert 35; Zeiss). The number of samples in each case was determined using the progressive mean procedure [22], which assures a counting error of less than 5%.

The photosynthetic response was measured as the effective fluorescence quantum yield (Φ_{PSII}) in triplicates of experiments and controls using a ToxY-PAM fluorometer (Walz) at 72 h. Effective quantum yields were calculated as follows: $\Phi_{PSII} = (F'_m - F_t)/F'_m$, where F'_m and F_t are the maximum and steady-state fluorescence, respectively, of light-adapted cells [23].

The concentration causing 50% inhibition of growth and Φ_{PSII} at 72 h ($IC50_{(72)}$) in algae was evaluated according to the area-under-the-curve method prescribed by the International Organization for Standardization [24]. The $IC50_{(72)}$ values were determined by nonlinear regression analysis. Results are expressed as the mean ± standard deviation, and data are presented as the percentage inhibition of growth rate and Φ_{PSII} with regard to controls (unexposed cells).

Statistical analysis was performed using the computer software package GraphPad Prism (Ver 4.0; GraphPad Software).

Ability of microalgae to adapt to chromium

Massive cultures of *D. chlorelloides* were exposed previously to 25 mg/L of Cr(VI) (~2.5-fold the 100% inhibitory concentration obtained in the toxicity assays) and maintained in culture with chromium for three months to establish if some kind of resistant cells could arise when microalgae are exposed to lethal concentrations of heavy metals. When microalgal cultures are exposed to lethal doses of chromium, the sensitive cells die because of the toxic effect of Cr(VI). After further incubation for three months, however, the growth of some algal variants resistant to the toxic effect of Cr(VI) could be detected. Growth rate and photosynthetic yield of these chromium-resistant microalgae were determined (as described previously) and then compared with those of sensitive microalgae.

The key to understanding adaptation of microalgae so these organisms can endure a chromium-contaminated environment seems to be characterizing the algal variants that appear after the massive destruction of sensitive cells. Heavy metal-resistant cells could arise by two procedures: First, through acquired resistance during the exposure to chromium (physiological adaptation), and second, through spontaneous mutations occurring randomly before chromium exposure (genetic adaptation). A modified fluctuation analysis [25] was performed as described previously by López–Rodas et al. [26] and Costas et al. [20] to distinguish between chromium-resistant cells arising through acquired physiological adaptation during exposure to Cr(VI) and chromium-resistant cells arising as result of rare, spontaneous mutations occurring before Cr(VI) exposure.

The fluctuation value was estimated as follows: Fluctuation $= CV_{set\ 1}/CV_{set\ 2}$, where CV is the variance in the number of cells per culture divided by the mean variance in the number of cells per culture, set 1 is the experimental culture, and set 2 is the control culture. A fluctuation value of approximately one indicates that chromium resistance is a consequence of physiological adaptation, and a fluctuation value of greater than one indicates that chromium resistance is a consequence of genetic mutations. The mutation rate (µ) was calculated as $\mu = -L_e P_0/(N_t - N_0)$, where $\log_e$ is the natural algorithm, P_0 is the proportion of cultures showing no resistant cells, N_t is the cell number at the end of the experiment, and N_0 is the cell number at the start of the experiment.

RESULTS

The Cr(VI) showed acute toxicity to sensitive microalgae, inhibiting both cell growth and $\Phi_{(PSII)}$, with $IC50_{(72)}$ values of 1.64 and 1.54 mg/L, respectively (Table 1). The concentration-

Table 1. Comparison of 72-h median inhibition concentrations ($IC50_{(72)}$) for growth and photosynthetic response (Φ_{PSII}) and associated 95% confidence limits (CL) for sensitive and resistant populations of *Dictyosphaerium chlorelloides* exposed to Cr(IV)[a]

		Growth inhibition (mg/L)		$\Phi_{(PSII)}$ inhibition (mg/L)	
Population	*n*	$IC50_{(72)}$	95% CL	$IC50_{(72)}$	95% CL
Sensitive	8	1.64	1.43–1.98	1.54	1.29–1.89
Resistant	8	20.61*	19.57–21.66	17.26*	15.04–19.48

[a] An asterisk indicates a significant difference ($p < 0.05$) with respect to values obtained for sensitive populations.

response of growth inhibition was similar to the concentration-response of $\Phi_{(PSII)}$ inhibition (Figs. 1 and 2, respectively).

When *D. chlorelloides* cultures were exposed to a 25 mg/L of Cr(VI) (~2.5-fold the 100% inhibitory concentration), these organisms became light in appearance after some days because of destruction of the sensitive cells by the toxic effect of Cr(VI). After further incubation for three months, however, some cultures became colored again because of growth of a rare algal variant that was resistant to the effect of Cr(VI). These resistant cells are on the order of 12-fold more resistant compared with those sensitive cells from Cr(VI)-free cultures. The concentration-response to Cr(VI) growth rate and $\Phi_{(PSII)}$ confirm that the resistant algae obtained from cultures with long-term Cr(VI) exposure developed a higher tolerance to this heavy metal. Comparison of $IC50_{(72)}$ values showed statistically significant differences ($p < 0.05$) between the wild-type, sensitive algae and the rare, resistant algal variants (Table 1).

The key to understanding adaptation of microalgae so that these organisms can persist in a chromium-contaminated environment seems to be characterizing these algal variants that appear after massive destruction of the sensitive cells. The fluctuation analysis has demonstrated that most cells of each experimental culture of set 1 (experimental) and set 2 (control) died because of the toxic effect of chromium. After further

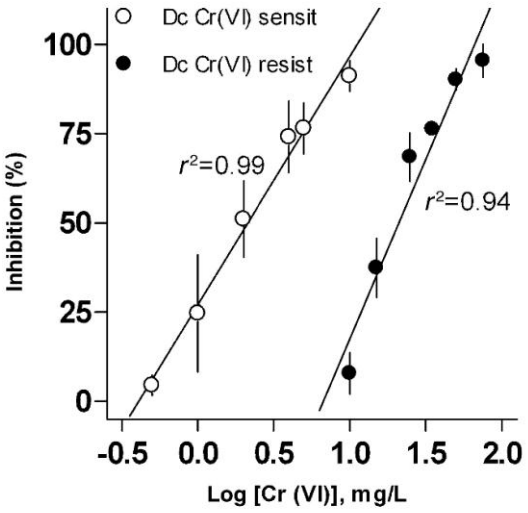

Fig. 1. Growth inhibition response induced by Cr(VI) exposure in sensitive (○) and resistant (●) *Dictyosphaerium chlorelloides* populations. Points represent the mean, with vertical lines showing the standard deviation ($n = 8$).

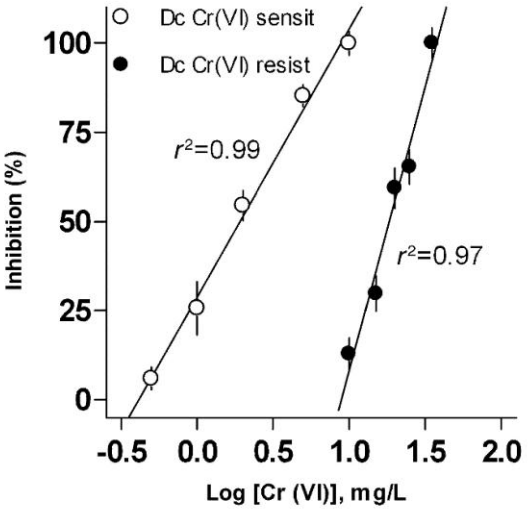

Fig. 2. Photosynthetic inhibition response measured as the effective fluorescence quantum yield (Φ_{PSII}) as induced by Cr(VI) exposure in sensitive (○) and resistant (●) *Dictyosphaerium chlorelloides* populations. Points represent the mean, with vertical lines showing the standard deviation ($n = 8$).

incubation for several weeks, however, some cultures of *D. chlorelloides* had again increased their density, apparently because of growth of a Cr(VI)-resistant algal variant. In set 1, a high fluctuation (from 0 to $>10^5$ resistant cells/culture flask) was observed after 60 d of Cr(VI) exposure. By contrast, nearly 1.5×10^5 Cr(VI)-resistant cells were detected in all cultures from set 2. In addition, a low Cr(VI)-resistant cell fluctuation was observed in set 2 (variance/mean ~0.2), indicating that the high fluctuation found in set 1 cultures was caused by processes other than sampling error. As in set 1 cultures, the variance significantly exceeded the mean (variance/mean ~2.5), so it could be inferred that Cr(VI)-resistant cells have arisen by rare, preselective, spontaneous mutations rather than by physiological adaptation or postselective mutations appearing in response to Cr(IV) (Table 2).

The estimated μ value of Cr(VI)-sensitive organisms to Cr(VI)-resistant organisms in *D. chlorelloides* was calculated as 1.77×10^{-6} mutants per cell division (Table 2). Isolated Cr(VI)-resistant mutants growing in absence of Cr(VI) have shown a small diminution of growth rates with respect to those found in Cr(VI)-sensitive cells. The coefficient of selection (*s*) of Cr(VI)-resistant mutants was 0.125. By using μ and *s* values,

Table 2. Fluctuation analysis of *Dictyosphaerium chlorelloides* exposed to 25 mg/L of Cr(VI)[a]

	Set 1	Set 2
No. of replicate cultures	105	30
No. of cultures containing the following no. of Cr(VI) resistant cells per milliliter		
0	86	0
$<1.5 \times 10^5$	7	0
$>1.5 \times 10^5$	12	30
Fluctuation	13.05	
Mutants per cell division (μ)	1.77×10^{-6}	

[a] Set 1 is the experimental culture, and set 2 is the control culture.

the frequency of Cr(VI)-resistant alleles was estimated as 14 resistant cells per 10^{-6} wild-type cells.

DISCUSSION

The standard toxicological analysis clearly demonstrates that microalgae are very sensitive to the effect of chromium. The $IC50_{(72)}$ obtained with Cr(VI) exposures in *D. chlorelloides* is in agreement with those obtained by other authors using toxicity test assays with marine [27] and freshwater [28] algal species. In spite of this, evidence in the literature demonstrates the existence of some kind of algal interspecies variability for Cr(VI) sensitivity [29,30].

Subsequently, it was verified that after destruction of most algae following a chromium exposure, some rare, resistant algal variants are able to grow in an environment with chromium levels approximately 26-fold higher than those that destroy the sensitive, wild-type algae. Apparently, resistance of algae to chromium seems to be higher than that found in other aquatic organisms [9]. Because only algae previously exposed to chromium seem to be resistant to treatments with this metal, it has been proposed that the reduction of toxic effects of chromium results from interaction among chromium, algal-excreted organic molecules, and the algal cell surface [31]. Microalgae and related eukaryotic photosynthetic organisms have preferentially developed the production of peptides capable of binding heavy metals [32–34]. This complex interaction between chromium and algal excretions takes place only after the algae have been stressed by a previous contact with the metal.

The present results unequivocally demonstrate that chromium-resistant algal variants also occur within clonal cultures that have not been exposed previously to chromium. Consequently, chromium resistance may be explained in terms of physiological adaptation, mainly as a consequence of changes in gene expression [13] or genetic adaptation as a result of rare mutations that occurred within the clonal cultures before chromium exposure [14]. The key to understanding the adaptation of unexposed algae to the extremely adverse effects of chromium is to analyze the rare algal variants that occur after the massive destruction of sensitive cells by Cr(VI). Fluctuation analysis is the appropriate procedure to discriminate between chromium-resistant algae arising by rare, spontaneous mutations occurring randomly during replication of organisms before exposure to this selective agent and chromium-resistant algae arising through specifically acquired adaptation induced by chromium exposure. The large fluctuation observed in the number of chromium-resistant cells in set 1, in contrast to the insignificant fluctuation observed in set 2, unequivocally demonstrates that resistant algal variants arose by a rare, spontaneous, single mutation that occurred before exposure to geothermal waters and not through physiological adaptation in response to Cr(VI). Exposure did not stimulate the appearance of resistant cells at all. Accordingly, algae can rapidly adapt to chromium contamination by single mutations. Recent evidence suggests that mutation at one loci can achieve adaptation of algae to other very hostile, heavy metal-enriched, natural environments [35–37].

The mutation rate from Cr(VI)-sensitive organisms to Cr(VI)-resistant organisms in *D. chlorelloides* (1.77×10^{-6} mutants/cell division) was in the middle range of the mutation rates (from 2.1×10^{-5} to 2.7×10^{-7} mutants/cell/generation) described in algae for resistance to complex mixtures of heavy metals from several extreme, natural environments [11,35–37]. The presence of Cr(VI)-resistant cells in populations of *D. chlorelloides* is caused by a rare, spontaneous mutation that occurs before Cr(VI) exposure. New resistant mutants arise in each generation. However, most of these mutants are eliminated, because Cr(VI)-resistant mutants are detrimental to species fitness in the absence of chromium contamination. At any one time, the balance between the continuous appearance of mutants and their selective elimination determines the number of remaining, chromium-resistant mutants in algal populations growing in the absence of chromium (i.e., this may be the case for wild-type populations developing in nonpolluted waters). Therefore, a natural population would be constituted predominantly by wild-type, chromium-sensitive genotype cells and, at the same time, in a very small fraction by a clone line of chromium-resistant mutant cells (i.e., in an experimental population at any given time are ~14 resistant mutants per 10^6 sensitive, wild-type cells).

Finally, the present results tentatively indicate that the different response in photosynthetic activity observed between sensitive and resistant cells of *D. chlorelloides* in the presence of Cr(VI) could be used to obtain a chromium-specific microalgal biosensor. Hexavalent chromium and other heavy metals in water usually are analyzed with time-consuming techniques that require laboratory hardware and that are not suitable for in situ, continuous monitoring [38]. As a result, many efforts have been made to develop biosensors for continuous monitoring of Cr(IV) [39]. Unfortunately, microalgal biosensors are not specific. Altamirano et al. [40], however, propose a new genetic approach for increasing the specificity of microalgal biosensors based on the use of a sensitive genotype (sensitivity) and a resistant mutant (specificity). In this sense, it should be possible to use the differential response in photosynthetic activity of sensitive and resistant cells of *D. chlorelloides* in the presence of Cr(VI) for continuous monitoring of this specific pollutant in water. The biosensor could be based on the joint use of two different genotypes to detect chromium: The sensitive Cr^s to obtain sensitivity, and the resistant Cr^r mutant to obtain specificity.

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II.IV. Adaptation of green microalgae to herbicides simazine and diquat as result of pre-selective mutations

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Adaptation of green microalgae to the herbicides simazine and diquat as result of pre-selective mutations

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ABSTRACT

Aquatic ecosystems located close to agricultural areas are increasingly polluted by herbicides. We evaluated the capacity for adaptation of green microalgae to lethal concentrations of the herbicide simazine in one strain of *Dictyosphaerium chlorelloides* and two strains of *Scenedesmus intermedius*, as well as adaptation to the herbicide diquat in one of the strains of *S. intermedius*. A Luria–Delbrück fluctuation analysis was carried out in order to distinguish between resistant cells arising from physiological adaptation (acclimatization) or post-adaptive mutation (both events occurring after the exposure to the herbicides), and adaptation due to mutations before the exposure to the herbicides. Simazine-resistant cells arose by rare spontaneous mutations before the exposure to simazine, with a rate of 3.0×10^{-6} mutants per cell per generation in both strains of *S. intermedius*, and of 9.2×10^{-6} mutants per cell per generation in *D. chlorelloides*. Diquat-resistant cells in *S. intermedius* arose by pre-selective mutations with a rate of 17.9×10^{-6} per cell per generation. Rare, pre-selective mutations may allow the survival of green microalgae in simazine- or diquat-polluted waters, via herbicide-resistant selection. Therefore, human-synthesized pollutants, such as the herbicides simazine and diquat, could cause the emergence of evolutionary novelties in aquatic environments.

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1. Introduction

At present, global extinction rates of organisms are 50–500 times background and are increasing due to human activities that are altering biosphere-level processes (Woodruff, 2001). The biodiversity crisis is reasonably understood for multi-celled organisms, but little is known about organisms as abundant and ecologically important as microalgae and cyanobacteria, which are the base of trophic webs in aquatic ecosystems (Falkowski and Raven, 1997). One of the causes of the present biodiversity crisis is intensive agriculture, because it is supported by the massive use of compounds with biocidal properties (Tilman, 1999; Malato et al., 2001). Freshwater habitats close to agricultural areas are sinks for a large array of herbicides, so that phytoplankters are exposed to a multitude of these toxic compounds (Junghans et al., 2006). In fact, it is considered that herbicides are among the most significant human-synthesized pollutants in aquatic ecosystems (Koenig, 2001). The unwanted side effects of herbicides include the selection of non-target species and strains (Belfiore and Anderson, 2001; Palumbi, 2001). In fact, it has been proposed that the emergence of unpredictable evolutionary novelties (such as resistant-herbicide phytoplankters) could be a distinctive feature of the future biosphere (Myers and Knoll, 2001). Therefore, although phytoplankters usually experience local extinction in herbicide-polluted waters they can also develop two different possibilities to survive the harmful effects of herbicides: new genetic variants originating by spontaneous mutation can be selected (genetic adaptation) (Sniegowski and Lenski, 1995; Belfiore and Anderson, 2001; Sniegowski, 2005), or else gene expression can be modified (physiological adaptation, also called acclimatization; Bradshaw and Hardwick, 1989). However, some evolutionary studies in bacteria (Cairns et al., 1988; Foster, 2000; Roth et al., 2006) and yeasts (Heidenreich, 2007) have suggested that adaptive mutations could be a process resembling Lamarckism which, in the absence of lethal selection, produces mutations that relieve selective pressure. Therefore, the key to this debate is to know whether the adaptation process allowing phytoplankters to survive and proliferate in herbicide-polluted waters appears before or after the exposure of the cells to the herbicide. Fig. 1 shows the different adaptive possibilities for phytoplankton in herbicide-polluted waters. In this framework, we recently demonstrated that the freshwater cyanobacterium *Microcystis aeruginosa* could proliferate in algaecide copper-polluted (García-Villada et al., 2004) or the herbicide glyphosate-polluted (López-Rodas et al., 2007)

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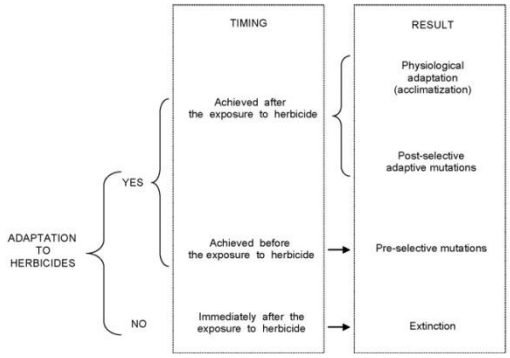

Fig. 1. Adaptation strategies of phytoplankton in herbicide-polluted waters.

waters, via pollutant-resistant clone selection originating from pre-selective mutations.

In order to improve our understanding of the adaptation process involved in the survival and proliferation of phytoplankters in herbicide-polluted waters, we addressed the adaptive mechanism of freshwater chlorophyceans to lethal doses of the herbicides simazine and diquat. By using the experimental procedure called fluctuation analysis (Luria and Delbrück, 1943), we were able to demonstrate if the adaptation to lethal herbicide concentrations could take place in wild-strains of phytoplankters and, secondly, to discriminate between acquired adaptations in response to the herbicides (by acclimatization or putative adaptive mutations; the first case is not an evolutionary event) and resistant cells arising from rare spontaneous mutations that appear prior to the herbicide exposure. We demonstrate the occurrence of very rapid evolution in three strains of chlorophyceans as result of pre-selective mutations conferring herbicide-resistance.

2. Materials and methods

2.1. Experimental organisms and culture conditions

The experiments were performed with three different strains of chlorophyceans from the algal culture collection of the Genetics Laboratory, Veterinary Faculty, Complutense University (Madrid, Spain). The SiM strain of *Scenedesmus intermedius* Chodat was isolated from Entreuka pond in the Sahel desert (Mauritania), where herbicides have never been used, whereas the SiD strain was isolated from a lagoon in Doñana National Park (SW Spain), a place sometimes exposed to runoff from nearby agricultural areas. The strain Dc of *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman was isolated from a high-mountain, pristine lagoon in Sierra Nevada National Park (SE Spain).

The strains were grown axenically in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma–Aldrich Chemie, Taufkirchen, Germany), at 20 °C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Strains were maintained in mid-log exponential growth by serial transfers of a cell inoculum to fresh medium (details in Carrillo et al., 2003). Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures. All three strains were used in the study of adaptation to simazine, whereas the adaptation to diquat was exclusively studied with strain SiD.

2.2. Toxicity test: effect of simazine and diquat on growth rate

We selected herbicides used at the present (diquat) or in the recent past (simazine) in the control of aquatic plants and algae (http://www.epa.gov). The toxic effect of simazine (2-chloro-4,6-bis[ethylamino]-*s*-triazine) and diquat (1,1′-ethylene, 2,2′-bipyridyl) on growth rate of the wild-type strains was assessed as follows. A stock solution of simazine was prepared in BG-11 medium with addition of 0.5% dimethyl sulfoxide. Each experimental culture was inoculated with 1.5×10^5 cells from mid-log exponentially growing cultures and exposed to concentrations from 0 to 5 μg L^{-1} of simazine. A diquat stock solution was prepared in BG-11 medium to obtain serial dilutions of 10, 30, 60 and 110 μg L^{-1}, and the experimental cultures were inoculated with 5×10^6 cells from mid-log exponentially growing cultures. The herbicides were purchased from Sigma–Aldrich Chemie (Taufkirchen, Germany). Three replicates of each concentration of both herbicides were prepared, as well as three unexposed controls.

The toxic effect of the herbicides was estimated by calculating acclimated maximal growth rate (m) in mid-log exponentially growing cells, in the presence of different concentrations of herbicide, by using the equation of Crow and Kimura (1970):

$$m = \frac{\log_e(N_t/N_0)}{t}, \quad (1)$$

where N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively, and t = 7 d, the time that cultures were exposed to different doses of simazine or diquat.

Experimental cultures and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a haemocytometer and an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany).

2.3. Fluctuation analysis of the transformation herbicide-sensitive → herbicide-resistant

A modified Luria–Delbrück analysis for application to liquid cultures with microalgae (Costas et al., 2001; López-Rodas et al., 2001) was used to investigate the origin of herbicide-resistant cells. The modification of the analysis involves the use of liquid medium containing the selective agent rather than plating on a solid medium, as was done by Luria and Delbrück (1943) with bacterial cultures.

Two different sets of experimental cultures were prepared. In the first set (set 1), 100–103 (for simazine) or 90 (for diquat) 20 mL culture flasks, containing 10 mL of BG-11 medium, were inoculated with $N_0 = 10^2$ cells (a number small enough to reasonably ensure the absence of pre-existing mutants in the strain). In the study with simazine, when each culture reached $N_t = 10^5$ cells, it was supplemented with a lethal dose (determined as explained in Section 2.2) of 3.1 μg L^{-1} simazine. The experiment with diquat started with $N_t = 6.2 \times 10^5$ cells of SiD cells, then the cultures were supplemented with a lethal concentration (determined as explained in Section 2.2) of 120 μg L^{-1} diquat. For set 2 (set control), 30 aliquots of 10^5 (for simazine) or 6.2×10^5 (for diquat) cells from the same parental population were separately transferred to culture flasks containing fresh liquid medium with the herbicide at the same concentration as set 1 cultures. Both herbicides are easily sorbed by organic matter (http://extoxnet.orst.edu/ and http://pesticide/org/) and half-life for simazine and diquat in water are 30 and 2 d, respectively. Consequently, in order to maintain lethal concentrations of herbicides in the cultures, they were centrifuged to form a pellet of cells in the tube, the medium was decanted and 10 mL of fresh liquid medium with the herbicide was added every fifth day. All cultures were kept under selective conditions and observed after 60 d, a period of time long enough to

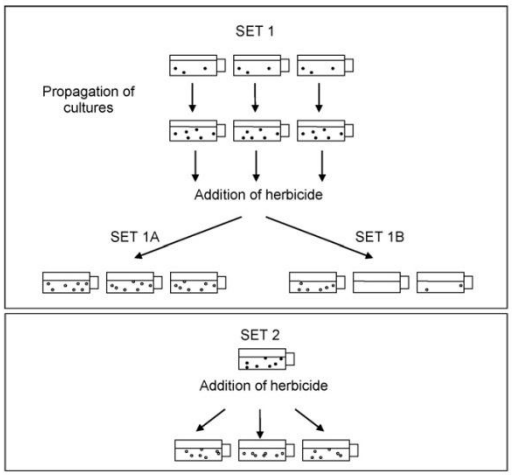

Fig. 2. Schematic diagram of the modified Luria and Delbrück (1943) fluctuation analysis. In the set 1, several cultures each inoculated with small inoculums were propagated until a high cell density was reached, and then a lethal dose of herbicide was added. If resistant cells arose by acclimatization or post-adaptive mutations (see Fig. 1) the number of resistant cells in all the cultures must be similar (set 1A). If adaptation is achieved by rare mutations (see Fig. 1) occurring in the period of the propagation of cultures the difference of the number of resistant cells in each culture must be huge (set 1B). Set 2 samples the variance of parental populations as an experimental control. In this case, the number of resistant cells in all the cultures must be similar. Black dots represent herbicide-sensitive cells while herbicide-resistant cells are shown by white dots.

allow resistant cells to grow. At the end of the experiments, the number of resistant cells in both sets was counted. The cell count was performed by at least two independent observers.

According to Luria and Delbrück (1943), two different results can be found in set 1 when conducting a fluctuation analysis, each of them being interpreted as the independent consequence of two different phenomena of adaptation. If resistant cells arose after the exposure of the cells to the selective agent (by acclimatization or specific post-selective mutations) the variance in the number of cells per culture would be low (Fig. 2, set 1A) because every cell is likely to have the same chance of developing resistance. Therefore, the coefficient of variation (CV = SD × 100/mean) of the number of resistant cells per flask must be relatively low. By contrast, if cells appeared by random, pre-selective mutations occurring before the exposure to the selective agent, high variation in the inter-culture number of resistant cells should be found (Fig. 2, set 1B) and, consequently, the CV should be relatively high. Obviously, another result (0 resistant cells in each culture) could also be found, indicating that neither selection on spontaneous mutations that occur prior to herbicides exposure, nor specific adaptation during the exposure to herbicides, took place.

Set 2 (Fig. 2) is the experimental control of the fluctuation analysis. It samples all the sources of variance associated with the experimental procedure. Thus, despite the way resistant cells appear, inter-culture (flask-to-flask) variance of resistant cells in set 2 should be similar to the average of resistant cells in set 2 cultures. Moreover, if a similar CV value is found in sets 1 and 2, it confirms that resistant cells appeared by adaptive mutations or acclimatization (i.e. after the exposure to the herbicide). By contrast, if the CV from set 1 is significantly higher than the CV in set 2, it means that resistant cells arouse by spontaneous mutations prior to exposure to herbicides. The comparison of CVs was performed by the one-tailed *Z*-test according to Zar (1999).

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. There are different approaches for accomplishing this estimation (Rosche and Foster, 2000). Due to methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of cultures from set 1 showing no resistant cells (P_0 estimator; Luria and Delbrück, 1943) was used to calculate the mutation rate (μ) by using the equation:

$$\mu = -\frac{\log_e P_0}{N_t - N_0} \quad (2)$$

2.4. Mutation-selection equilibrium

If the mutation from wild-type, herbicide-sensitive allele to herbicide-resistant allele is recurrent and, in addition, the herbicide-resistant allele is detrimental in fitness in the absence of herbicides, most of these mutants are eliminated sooner or later by natural selection, if not by chance. At any one time, there will be a certain number of cells that are not yet eliminated. The average number of such mutants will be determined by the balance between μ and the rate of selective elimination, in accordance with the equation from Kimura and Maruyama (1966):

$$q = \frac{\mu}{\mu + s}, \quad (3)$$

where q is the frequency of the herbicide-resistant allele and s is the coefficient of selection, calculated as follows:

$$s = 1 - \left(\frac{m_S^r}{m_S^s}\right), \quad (4)$$

where m^r and m^s are the acclimated maximal growth rates of herbicide-resistance and herbicide-sensitive cells measured in non-selective conditions (i.e. in BG-11 medium), respectively.

3. Results

The exposure to simazine had a toxic effect on the three algal strains evaluated. A concentration of 1.5 $\mu g\,L^{-1}$ simazine inhibited the growth of the three strains totally and the cultures became clear after some days by massive destruction of the sensitive cells. Similarly, a concentration of 60 $\mu g\,L^{-1}$ of diquat was lethal for SiD cells. In accordance with these lethal concentration figures, concentrations of 3.1 $\mu g\,L^{-1}$ simazine and 120 $\mu g\,L^{-1}$ diquat were selected for the fluctuation analysis tests.

The fluctuation analysis culture flasks were incubated for 2 months. After this time, cell growth appeared in some culture flasks in the four experiments carried out. A high fluctuation in the number of resistant cells per culture flask (from 0 to $>10^5$) was found in set 1 of the three strains of chlorophyceans tested against simazine (Table 1). The CVs of the set 1 experiments were significantly ($P < 0.001$) higher than those found in the respective set 2 controls (Table 1). Similarly, the number of diquat-resistant cells of SiD per flask ranged from 0 to $>10^5$ in set 1, whereas the figure ranged from 10^3 to 10^4 in set 2 (Table 2). The CV from set 1 was significantly ($P < 0.001$) higher than that from set 2 (Table 2). Consequently, the high fluctuation found in set 1 cultures in the four experiments should be due to processes other than sampling error, and it could be inferred that herbicide-resistant cells arose prior to herbicide exposure by rare, spontaneous mutations rather than by specific adaptation (i.e. acclimatization) during herbicide exposure.

The spontaneous mutation rate (μ) of simazine-sensitive cells to simazine-resistant cells, using the P_0 estimator, was estimated at 3.0×10^{-6} mutations per cell per generation in both strains of *S. intermedius*, and at 9.2×10^{-6} mutations per cell per generation in *D. chlorelloides* (Table 1). In the case of diquat, the value of μ in the

Table 1
Fluctuation analysis of simazine-resistant variants in the green microalgae *Scenedesmus intermedius* (strains SiM and SiD) and *Dictyosphaerium chlorelloides* (strain Dc).

	Strain SiD		strain SiM		strain Dc	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
No. of replicate cultures:	100	30	102	30	103	29
No. of cultures containing the following no. of simazine-resistant cells:						
0	74	0	75	0	41	0
$1–10^3$	0	0	1	0	1	0
$10^3–10^4$	1	0	0	0	0	0
$10^4–10^5$	2	0	10	0	16	0
$>10^5$	23	30	16	30	45	30
CV of the no. simazine-resistant cell per replicate (%):	106.3	42.6	239.2	26.2	375.0	18.3
μ (mutants per cell per generation)	3.0×10^{-6}		3.0×10^{-6}		9.2×10^{-6}	

Table 2
Fluctuation analysis of diquat-resistant variants in the green microalgae *Scenedesmus intermedius*, strain SiD.

	Set 1	Set 2
No. of replicate cultures:	90	30
No. of cultures containing the following no. of diquat-resistant cells:		
0	30	0
$1–10^3$	5	0
$10^3–10^4$	28	30
$10^4–10^5$	21	0
$>10^5$	6	0
CV of the no. diquat-resistant cell per replicate (%):	104.7	12.2
μ (mutants per cell per generation):	17.9×10^{-6}	

strain SiD was one order of magnitude higher (17.9×10^{-6} mutations per cell per generation) than those found for the resistance against simazine (Tables 1 and 2).

The simazine-resistant cells, in absence of this herbicide, showed lower growth rate values than simazine-sensitive cells. The coefficients of selection (s) were estimated to be 0.109, 0.267 and 0.306 in strains SiD, SiM and Dc, respectively. The frequency (q) of simazine-resistant alleles, in wild-type populations in the absence of simazine, was calculated by using the values of s and μ: 27.5, 11.2 and 30.0 simazine-resistant per 10^6 wild-type cells, in strains SiD, SiM, and Dc, respectively. In the case of diquat, the computed q value (by using a derived s value of 0.215) was of 83.2 resistant cells per 10^6 wild-type SiD cells.

4. Discussion

In freshwater habitats close to agricultural areas phytoplankters are exposed to a large array of herbicides (Junghans et al., 2006). If the concentration of herbicides exceeds the lethal one for the sensitive, wild-strains of phytoplankters the immediate effect will be its local extinction but the arising of unpredictable evolutionary novelties (such as resistant-herbicide strains) could also occur (Myers and Knoll, 2001). In this work, we present an experimental model of evolution to analyze adaptation of phytoplankters to survive in herbicide-polluted aquatic environments. In particular, the possible adaptation of chlorophyceans to lethal concentrations of the herbicides simazine and diquat was addressed. When chlorophycean cultures were exposed to the herbicides, they became clear after some days due to total growth inhibition and subsequent massive destruction of the cells by the lethal effect of herbicides. However, if they were further incubated some cultures became green again, due to the growth of variants that were resistant to the herbicides. By using the statistical and experimental approach named fluctuation analysis (Luria and Delbrück, 1943), we were able to discriminate between simazine- or diquat-resistant cells arising by rare spontaneous mutation occurring randomly during propagation of organisms under non-selective conditions, and herbicide-resistant cells arising by adaptive mutations or though acclimatization in response to the herbicides. The high fluctuation in the number of herbicide-resistant cells observed in set 1 cultures, in contrast with low fluctuation of set 2 controls, shows that herbicide-resistant cells have arisen from rare, pre-selective spontaneous mutations occurring randomly during replication of organisms prior to exposure to the herbicides.

The mutation rates from simazine-sensitivity to simazine-resistance in the three stains of microalgae ($3.0–9.2 \times 10^{-6}$ mutants per cell per generation) were found to be in the middle of the range of the mutations rates (from 2.1×10^{-5} to 2.7×10^{-7} mutants per cell per generation) we have described in cyanobacteria and microalgae, for resistance to many other biocides and extreme natural environments (Costas et al., 2001; López-Rodas et al., 2001; Baos et al., 2002; García-Villada et al., 2002, 2004; Flores-Moya et al., 2005; Costas et al., 2007, 2008; López-Rodas et al., 2007, 2008a,b,c, 2009). However, diquat-resistant cells appear spontaneously in wild populations with a frequency one order magnitude higher (17.9×10^{-6}) than simazine-resistant cells.

Since mutation is recurrent in each generation, new mutant cells are arising continuously. Herbicide-resistant mutants are impaired in growth rate and, consequently, most of them are eventually eliminated by natural selection (Crow and Kimura, 1970; Spiess, 1989). At any given time, the balance between the continuous appearance of mutants and their selective elimination determines the number of remaining herbicide-resistant mutants in algal populations growing in the absence of herbicides. This may be the case in wild-type populations developing in non-polluted waters. Consequently, the population would be predominantly a clone line of simazine- or diquat-sensitive genotypes, accompanied by, as a very small fraction, clone lines of simazine- or diquat-resistant mutants. Moreover, recurrent exposures to herbicides could cause the rise of strains with higher selection coefficients and, consequently, enhancing the frequency of the resistance-alleles as the consequence of mutation-selection equilibrium. This is illustrated in the case of *S. intermedius*: the mutation rate from simazine-sensitivity to simazine-resistance was similar in two strains of this species. However, the frequency of the resistant allele, in the absence of simazine, was 2.5 times higher in the strain isolated from Doñana National Park (where herbicides sometime arrive in runoff) than in the strain isolated from the pristine pond Entreuka in the Sahel desert. This difference is based on a higher selection coefficient in the strain SiM than in the strain SiD.

5. Conclusions

Rare spontaneous mutations conferring resistance against simazine or diquat seem to be enough to assure survival of microalgae populations in simazine- or diquat-polluted waters. Moreover,

in a hypothetical future scenario with herbicide-polluted waters, the primary production supported by phytoplankters could be significantly lower than that in the present, as a consequence of the diminished growth rate of resistant mutants in comparison to wild-type cells.

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CAPITULO III

DETERMINACIÓN DE LA MÁXIMA CAPACIDAD DE ADAPTACIÓN

"*Adapt or perish, now as ever, is nature's inexorable imperative*".

(H.G.Wells. Filósofo y novelista británico)

En el presente capítulo se estudia la capacidad adaptativa de distintas especies fitoplanctónicas ante diversos ambientes selectivos. Mediante la utilización del protocolo de Ratchet se examina la máxima capacidad de adaptación de diversas especies y grupos fitoplanctónicos expuestos a distintas condiciones selectivas. Asimismo se analiza la capacidad de adaptación deferencial de cada uno de ellos en función de su naturaleza y distribución geográfica.

El capítulo se estructura en tres trabajos que analizan distintos agentes de selección:

- En el primer artículo se estudia la capacidad de adaptación al herbicida Simazina, el cual ha sido aplicado de forma abundante en agricultura para el control de las malas hierbas (García-Valcárcel y Tadeo 1999; Gunasekara *et al.* 2007) debido a su capacidad para la inhibición fotosintética (Quimbi *et al.* 1978). Este herbicida es ampliamente detectado en aguas tanto superficiales como subterráneas de distintos países desarrollados (Troiano *et al.* 2001). Residuos de este herbicida dan lugar a la contaminación de los sistemas acuáticos, tanto dulces como marinos, por lo que los organismos fitoplanctónicos se ven enfrentados a esta nueva sustancia. Para asegurar su persistencia deberán desarrollar distintos mecanismos de supervivencia que

serán asegurados en mayor o menor medida según la capacidad adaptativa de cada especie. Por ello, en el presente trabajo se examina la capacidad de adaptación ante esta sustancia de 13 especies fitoplanctónicas distintas seleccionadas según su hábitat específico y grupo taxonómico al que pertenecen.

- En el segundo artículo se analiza la capacidad adaptativa ante el metal pesado cobre en forma de Sulfato de Cobre. Esta sustancia ha sido ampliamente utilizada para el control de crecimientos algales en agua dulce (Elder y Horne 1978) fundamentalmente para la prevención de la proliferación de los blooms indeseables de la cianobacteria *Microcystis aeruginosa.* El uso de este alguicida ha sido prohibido en determinados países en los últimos años debido a los efectos tóxicos que produce. El presente trabajo analiza la máxima capacidad de adaptación de distintas especies fitoplanctónicas ante esta sustancia. Para ello se han elegido tres cepas de la cianobacteria *Microcystis aeruginosa y* dos especies de clorofitas (Scenedesmus intermedius) y *Dictyosphaerium chlorelloides*, como representantes de organismos eucariotas de agua dulce abundantes en embalses y pantanos.
- El último agente selectivo estudiado es el aumento de la temperatura. Hemos seleccionado un agente físico, ya que el aumento de temperatura determina una alteración en la dinámica de las poblaciones, derivada de cambios en el metabolismo y alteraciones de la sucesión espacial y temporal de especies (Hughes 2000). Por ello, mediante la determinación de la máxima capacidad de adaptación y la capacidad diferencial de las distintas especies ante el fenómeno de calentamiento global se podrían determinar las especies de fitoplancton más resistentes y elucidar la distribución futura de las poblaciones. En este estudio se utilizaron las mismas especies que en el primer artículo, con objeto de determinar de nuevo, si el hecho de proceder de distintos hábitats o la naturaleza de cada especie en cuestión podría explicar diferencias en la capacidad adaptativa ante el fenómeno de calentamiento.

Los resultados encontrados en los tres capítulos muestran distintos grados de tolerancia según las especies analizadas así como una capacidad inter-específica para la adaptación genética.

III.I. Estimating the capability of different phytoplankton groups to adapt to contamination: herbicides will affect phytoplankton species differently

Research

New Phytologist

Estimating the capability of different phytoplankton groups to adapt to contamination: herbicides will affect phytoplankton species differently

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Summary

- Investigating the differential capacity of the response of phytoplankton to human-induced environmental forcing has become a key issue to understanding further the future repercussions on the functioning of aquatic ecosystems.
- The initial tolerance to the widely dispersed herbicide simazine was measured in diverse phytoplankton species. An experimental ratchet system maintaining large populations of dividing cells (which ensures the occurrence of rare spontaneous mutations that confer adaptation) and a strong selection pressure (which ensures the preservation of such mutations within the population) was later applied to estimate the capability of different groups of phytoplankton to adapt to simazine.
- Initially, simazine doses between 0.05 and 0.15 ppm were able to inhibit 100% growth in all the species tested. However, a significant increase in simazine resistance was achieved in all derived populations during the ratchet experiment. The differential capacity for simazine adaptation was observed among the different species.
- The capacity of different species to adapt to simazine can be explained in relation to taxonomic group, ploidy, growth rate and habitat preference. Haploid populations of continental Chlorophyta showed the greatest capacity to adapt to simazine. By contrast, populations of Haptophyta of open ocean regions were the group least capable of adapting to the herbicide.

Introduction

Changes in environmental conditions are occurring at an unprecedented rate as a result of large-scale changes caused by human activities. The massive loss of diversity, homogenization of biotas, proliferation of opportunistic species and unpredictable emergent novelties can be considered among the distinctive features of the future biosphere (Myers & Knoll, 2001). During the last century, the disappearance rate of species was estimated to be 500-fold higher than had been the case over the preceding centuries, giving rise to an annual extinction of 30 000 species out of the 11 million currently estimated (Woodruff, 2001).

Intensive agriculture, supported by the massive use of herbicides, pesticides and compounds with biocidal activity, is a significant cause of the biodiversity crisis (Tilman, 1999; Malato *et al.*, 2001). Fitness cost associated with the novel herbicide resistance alleles is important in evolutionary dynamics (reviewed by Vila-Aiub *et al.*, 2009). The impact of these toxic compounds on biodiversity threatens all ecosystems, being particularly significant in those characterized by a slow response to change, such as aquatic systems. The species collapse can be mainly attributed to the incapacity of organisms to cope with drastic environmental anthropogenic changes occurring in their habitats.

Freshwater reservoirs, lakes, rivers and the coastal ocean areas receive considerable amounts of anthropogenic pollutants, including a large number of herbicides, which are altering the chemical balance and biogeochemical cycles. These changes affect the totality of the aquatic biota, as they are exposed to unprecedented scenarios. However, phytoplankton can be highlighted as a likely target to experience this environmental forcing. Since these organisms represent the basis of the aquatic food web, the repercussions of the

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impact on phytoplankton populations will undoubtedly affect the rest of the components of the trophic web. Despite their microscopic size, phytoplankton are responsible for about half of the global primary production, driving essential biogeochemical cycles, exporting massive amounts of carbon to deep waters and sediments in the open ocean and strongly influencing the water–atmosphere gas exchanges (Rost *et al.*, 2008).

Phytoplankton includes 11 phyla of prokaryotic and eukaryotic microorganisms (Margulis & Schwartz, 1982). Some species are haploids, others are diploids, some grow rapidly and others grow slowly, some have recombination whole others are asexual. Becaue of this variety, the physicochemical shifts brought about by global change are expected to influence phytoplankton in numerous and various ways. It is well known that different taxa of phytoplankton have different environmental demands. Nutrient availability, light intensity, temperature, sinking, grazing and pathogen resistance are among the main conditions that decide competitive advantage and regulate species distribution. Hence, assuming the complexity of possible responses that may arise to cope with the varying habitat conditions, they still can be divided into adaptive and ecological reactions, such as variations in the rates of individual processes and shifts in species succession, respectively (Falkowski *et al.*, 1998; Boyd & Doney, 2002). Depending on which species or groups are affected and in what manner, variations have the potential to alter productivity and to cause feedback on biogeochemical cycles in many ways. Consequently, investigating the differential capacity of the response of phytoplankton to increasing amounts of pollution has become a key issue in understanding further the future repercussions on the functioning of aquatic ecosystems.

The capability of different groups of phytoplankton to adapt to strong herbicide selection pressure can be assessed experimentally. Although this approach is simplistic in terms of ecosystem interactions, the scientific importance of estimating the potential of phytoplankton to adapt to herbicide contamination is obvious. The application of rigorous experimental techniques as an approach to evolutionary ecotoxicological questions in marine and freshwater biology is novel and timely. Additionally, phytoplanktonic organisms possess a useful advantage for experimental evolutionary studies, as these organisms can be easily manipulated during numerous generations (reviewed in Flores-Moya *et al.*, 2008).

The majority of phytoplankton groups have been observed to have a great phenotypic plasticity to acclimate to modifications in environmental parameters and are able to survive in *a priori* adverse habitats as a result of physiological acclimation, which is supported by modifications of gene expression (Bradshaw & Hardwick, 1989; Fogg, 2001). However, when changes in environmental conditions exceed physiological limits, species survival will depend exclusively on adaptive evolution, which is in turn driven by the occurrence of mutations that confer resistance (Sniegowski & Lenski, 1995; Belfiore & Anderson, 2001). Previous studies on the adaptation of phytoplankton to both anthropogenic pollutants and extreme natural environments have demonstrated that physiological acclimation is optimally achieved under the least toxic conditions (Costas *et al.*, 2008; Carrera-Martinez *et al.*, 2010), whereas genetic adaptation is observed in waters ostensibly lethal for the experimental organisms (Flores-Moya *et al.*, 2005; Costas *et al.*, 2007; López-Rodas *et al.*, 2007, 2008a,b) and that this adaptation is usually attained as a consequence of single mutations (López-Rodas *et al.*, 2001; Costas *et al.*, 2001). Therefore, by analyzing the growth of different phytoplankton species belonging to diverse functional groups submitted to selection pressure during many generations, the differential specific adaptation capability can be assessed. Furthermore, this procedure allows for the occurrence of both pre-existing and/or arising mutations that may benefit the population.

In this work, experimental populations of 12 phytoplankton strains were exposed to intense short-term selection using an experimental protocol that maintains a strong selection pressure in a temporal scale up to several months over a very large population. The species tested represent different habitat-preference phytoplankton groups such as freshwater microalgae and cyanobacteria, coastal marine microalgae, and oceanic microalgae. These species were studied as a first step towards analyzing their different potentials for adaptation to anthropogenic-contamination processes.

In our experimental model, a selecting agent was chosen as a representative example for the current anthropogenic forcing: the simazine herbicide, which is widely dispersed and upon reaching the aquatic environment behaves as a major pollutant (http://www.epa.gov). Since s-triazine herbicides (as is the case of simazine) are specific inhibitors of photosynthetic electron transport (Bowyer *et al.*, 1990; Tietjen *et al.*, 1991), the primary producers represent the most susceptible component of the aquatic community. In fact, it is considered that herbicides are among the most significant human-synthesized pollutants in aquatic ecosystems (Koening, 2001). Phytoplankton is exposed to a multitude of herbicides mainly in aquatic habitats close to agricultural areas. Some of the unwanted side-effects of herbicides include the selection of nontarget species and strains (Palumbi, 2001), and the emergence of unpredictable evolutionary novelties such as herbicide-resistant phytoplankton (Marvá *et al.*, 2010).

Although, phytoplankton usually experience local extinction in herbicide-polluted waters, they can also survive the harmful effects of herbicides by some kind of adaptation. Even though this study might be considered an oversimplification of the reality, the evolutionary-ecological approach

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followed here constitutes a novel way to explore, through a rigorous experimental model, the differential capability of distinct functional phytoplankton groups to adapt to anthropogenic-induced changes in environmental conditions.

Materials and Methods

Experimental organisms and culture conditions

Twelve strains of eight phytoplankton species obtained from the Algal Culture Collection of the Universidad Complutense (Madrid, Spain) and belonging to three different functional phytoplankton groups (i.e. phytoplankton from continental freshwater, coastal marine waters and oceanic waters) were selected for the experiments.

The phytoplankton from inland water systems (PhI) included one strain of the Chlorophyta *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman (Dc), one strain of the Chlorophyta *Scenedesmus intermedius* Chodat (Si) and three strains of the Cyanobacteria *Microcystis aeruginosa* (Kützing) Lemmermann (MaD3, MaD6 and MaD7). *D. chlorelloides* was isolated from a pristine mountain lake in the Sierra Nevada (southwest Spain), whereas the other two species, *S. intermedius* and *M. aeruginosa*, were isolated from a pristine lagoon in Doñana National Park (southwest Spain). Neither site has had documented herbicide use in their proximities. Phytoplankton from coastal marine waters (PhC) made up the second group, which was formed by one strain of the Chlorophyta *Tetraselmis suecica* (Kylin) Butcher (Te) and one strain of the diatom (Bacillariophyta) *Phaeodactylum tricornutum* (Pt). The former was isolated from noncontaminated coastal waters of Sardinia (France), and the latter came from the northwestern waters of Spain. The third group constituted of phytoplankton from oceanic waters (PhO), which included marine Haptophyta, particularly three strains of *Emiliania huxleyi* (Lohm.) Hay et Mohler (ccmp 371, ccmp 372 and ccmp 373 from the UTEX stock) originally obtained from the Sargasso Sea, one strain of *Isochrysis galbana* Parke (Ig) and one strain of *Monochrysis lutheri* (Droop) Green (Ml), the latter two being isolated from the north central Atlantic.

All cultures were grown axenically in ventilated cell-culture flasks with filter cap (Greiner; Bio-One Inc., Longwood, NJ, USA) containing either 20 ml of BG11 medium (Sigma-Aldrich) for freshwater algae and cyanobacteria, or alternatively 20 ml of f/2 medium (Sigma) for marine algae. Flasks were placed at 22°C under a continuous photon flux density of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm provided by cool white fluorescent tubes. Cultures were maintained in mid-log exponential growth by serial transfers of a cell inoculum to fresh medium. Under these conditions, all the cultures grew asexually. Before experiments, cultures were recloned by isolating a single cell in order to avoid the inclusion of any previous spontaneous mutants that might have been accumulated in the culture.

Toxicity test: effect of simazine on growth rate of ancestral strains

The toxic effect of simazine (2-cloro-4,6-bis[ethylamino]-s-triazine) on growth rate of the 14 wild-type strains was assessed as previously described (Marvá *et al.*, 2010). Each experimental culture was inoculated with 1.5×10^5 cells from mid-log exponentially growing cultures and exposed to increasing concentrations of simazine (increase interval = 0.05 ppm). These chemicals were purchased from Sigma-Aldrich. Triplicates of each concentration were prepared, as well as three unexposed controls.

The toxic effect of the simazine was estimated by calculating acclimated maximal growth rate (m) in mid-log exponentially growing cells, in the presence of growing concentrations of herbicide, according to the following equation:

$$m = \log_e(N_t/N_0)/t, \qquad \text{Eqn 1}$$

(Crow & Kimura, 1970) where N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively, and $t = 5$ d is the time that cultures were exposed to increasing doses of simazine. Experimental cultures and controls were counted blind (i.e. the person counting the test did not know the identity of the tested sample), using a Beckman (Brea, CA, USA) Z2 particle counter. *Scenedesmus intermedius* (Si) were counted using a haemocytometer and an inverted microscope (Axiovert 35; Zeiss), since cell aggregation prevents reliability of results when using a particle counter.

Experimental design

Theoretical considerations Adaptive evolution depends exclusively on the occurrence of new mutations that confer resistance (reviewed by Sniegowski & Lenski, 1995). Consequently, it is feasible to estimate the maximal capability for adaptation of phytoplankton to a selecting agent through experimental models, which are able to maintain large populations of dividing cells, as this approach ensures both the probable occurrence of mutations that confer adaptation and the simultaneous maintenance of a strong selection pressure, which in turn ensures the preservation of such mutations within the population.

Unfortunately, it is quite difficult to estimate the optimum selection pressure that ensures enough incidences of mutations that confer adaptation, since strong selection pressures drastically reduce population size. This constraint can be overcome by performing experiments that include several values of selection pressure. To this end, Reboud *et al.* (2007) developed an experimental model aimed at

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evaluating the maximal potential for herbicide resistance evolution in *Chlamydomonas* based on the use of different herbicide concentrations, which thereby constituted selection pressures. Furthermore, Orellana *et al.* (2008) provided a modified procedure that allowed for maximizing the occurrence of mutants and their selection by applying variable selection pressures. Such an approach was designed to obtain microalgae with a maximum resistance to antibiotics, herbicides and heavy metals. A significant enhancement of this experimental procedure can be achieved by simply using different replicates of each strain under each selecting condition, which assures repeatability. These methodological approximations are referred to as ratchet assays.

Experimental setup In this work, an experimental system based on the ratchet protocols described previously by Reboud *et al.* (2007) and Orellana *et al.* (2008) was applied to estimate the maximal capability for adaptation of different phytoplankton species under increasing doses of the herbicide simazine. The protocol used here aims at reaching equilibrium between strong selection intensity, by means of ratcheting to increase herbicide dose, and the maintenance of a population size large enough to increase probability of rare spontaneous mutations that confer adaptation. These mutations occur randomly and not through specifically acquired adaptation included by simazine. Cultures must be ratcheted only up to a dose that supports population growth and are exposed to different selection levels. The experimental procedure was then applied in nine independent experiments (one for each phytoplankton species).

During the initial phase, three replicates of the control cultures containing growth medium and three replicates of cultures for each of the three initial doses of simazine treatments were prepared. The selected initial doses were identical for all groups and corresponded to 0.15, 0.45 and 1.5 ppm of simazine. Each replicate was grown in tubes of 5 ml capacity inoculated with 3×10^5 wild-type cells ml^{-1} that were obtained from mid-log exponential growing cultures, a number large enough to ensure a large final population. All cultures were counted using a Beckman Z2 particle counter (except *S. intermedius*, which was counted using a haemocytometer) and kept under the selecting conditions for 20 d and then observed. This period of time was long enough to allow control cultures to reach a high cell concentration. Under these conditions, cell concentrations were again counted and a comparison between control and experimental cultures was made. If a cell concentration in one of the treated tubes was the same or higher than that in control tubes, then the population had achieved noticeable growth. Subsequently, this population was ratcheted to the next cycle and subjected to a higher herbicide dose. Cultures not reaching a cell concentration similar to that found in control cultures were not transferred (Fig. 1).

Each herbicide ratchet cycle entails a threefold dose increase. The next cycles of the simazine treatment were selected as 4.5 ppm, 13.5 ppm and 40.5 ppm. It is worth noting that in this procedure, each individual tube is considered as an independent population. Therefore, if a tube belonging to the same initial triplicate tubes were to reach the growth observed in control cultures, it was ratcheted to the next cycle, regardless of whether the other two replicates did or did not achieve the same results. This criterion was understood to indicate different amounts of resistance, which can be attained separately, and resistant microalgae, which are likely to occur earlier in one tube with respect to the other two replicates exposed to the same dose. In other words, each tube presents a different random chance of particular beneficial mutations, which may then arise individually. Both control and treated cultures were again inoculated at this second stage with identical cell concentrations to those used during the first cycle.

A ratchet cycle was concluded when no further cell growth was observed in a specific replicate after a period of 100 d. The number of ratchet cycles was therefore species-dependent, as growth was a function of the ability to adapt to the selecting conditions.

The classic equations of Novick & Szilard (1950),

$$N_t = N_0 2^{t/T} \quad \text{Eqn 2}$$

$$g = t/T \quad \text{Eqn 3}$$

where N_t is the number of cells at time t, N_0 is the number of cells at time 0, t is the time, T is the generation time and g is the number of generations, were used to estimate the number of generations during the ratchet experiments.

Toxicity test: effect of simazine on growth rate of derived strains

Finally, the toxic effect of simazine on the growth rate of the 14 derived strains (obtained after the ratchet experiment) was assessed as previously described for wild-type strains before the ratchet experiment. The maximal herbicide dose that led to the growth of a derived strain obtained at the end of the ratchet cycles provides the estimate of maximum adaptation capacity of the aforementioned strain.

Results

Initially, the inhibitory doses of simazine were estimated in the 12 ancestral wild-type strains before the ratchet experiment (Table 1). Simazine doses between 0.05 and 0.15 ppm were able to inhibit 100% growth in all of the species tested. In spite of species diversity from different functional phytoplankton groups (i.e. from prokaryotic

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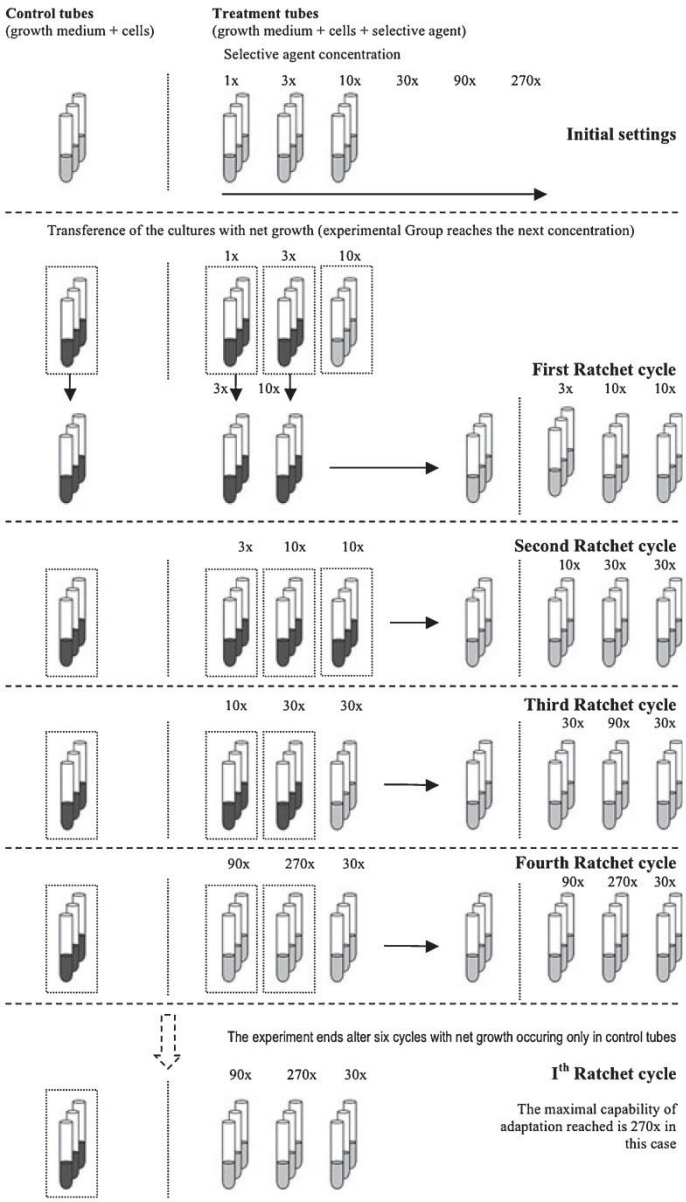

Fig. 1 Schematic representation of the ratchet experimental design. Three replicates of the control cultures and three replicates of cultures for each of the three initial doses of selected agent are present in each ratchet cycle. Each tube is transferred to the next concentration when the same net growth as the control tubes is reached (these tubes are represented as framed in the figure). Tubes that do not present net growth are maintained at the same concentration. A new ratchet cycle is considered each time the control tubes are transferred. The experiment ends after six cycles with net growth occurring only in the control tubes. At this point the maximal capability of adaptation corresponds to the maximal concentration of the selective agent that presents net growth.

inland water Cyanobacteria to oceanic Haptophyta), the inhibitory doses of simazine showed only a scarce degree of interspecific variation.

The potential for adaptation of the 12 strains was then estimated in derived populations obtained from ratchet experiments by continually maintaining large populations under strong selection pressure during the ratchet experiment. By contrast, considerable differences in the process of adaptation to simazine were seen among the diverse species and groups tested (Table 2). Despite this, differences in the time or in the number of generations required to achieve growth under increasing doses of simazine were observed among the different replicates of each strain (Table 2).

The oceanic phytoplankton group (Haptophyta species) showed the minimum adaptation capacity as compared with the rest of the groups. As indicated in Table 2, all the

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Table 1 Simazine concentration causing 100% growth inhibition measured in ancestral populations of various phytoplanktonic organisms before the ratchet experiments as well as in derived populations after the ratchet experiments

Strain	Ancestral populations (before the ratchet experiments)	Derived populations (after the ratchet experiments)	Increase in adaptation (after the ratchet experiments)
Oceanic microalgae			
Emiliania huxleyi (CCMP371)	0.10	0.15	1.5 times
Emiliania huxleyi (CCMP372)	0.10	0.15	1.5 times
Emiliania huxleyi (CCMP373)	0.05	0.15	Three times
Isochrysis galbana	0.10	0.15	1.5 times
Monochrysis lutheri	0.10	0.15	1.5 times
Coastal marine microalgae			
Tetraselmis suecica	0.15	1.5	10 times
Phaeodactylum tricornutum	0.10	0.45	4.5 times
Continental microalgae and cyanobacteria			
Scenedesmus intermedius	0.15	40.5	270 times
Dictyosphaerium chlorelloides	0.15	13.5	90 times
Microcystis aeruginosa (3D)	0.05	0.45	Nine times
Microcystis aeruginosa (6D)	0.05	0.45	Nine times
Microcystis aeruginosa (7D)	0.05	0.45	Nine times

These simazine concentrations were measured in triplicates of each strain inoculated with 1.5×10^5 cells from mid-log exponentially growing cultures and exposed to increasing simazine doses (increase interval = 0.05 ppm).

oceanic species were able to resist 0.15 ppm of simazine but growth was not observed above this dose. Therefore, 0.15 ppm was taken as the maximum concentration of simazine at which these microalgae could adapt.

The continental Chlorophyta species (*S. intermedius* and *D. chlorelloides*) exhibited the maximum capability for adaptation to the pollutant. Growth was detected in cultures of *S. intermedius* exposed to a simazine concentration as high as 40.5 ppm, while *D. chlorelloides* was able to sustain growth at 13.5 ppm (Table 2). By contrast, all the cyanobacteria strains were only able to adapt to 0.45 ppm of simazine (Table 2).

The two species of coastal microalgae selected show a distinct capability of adaptation. While *T. suecica* (Chlorophyta) was still able to grow at 1.5 ppm of simazine, cultures of the diatom *P. tricornutum* (Bacillariophyta) collapsed at doses of the herbicide higher than 0.45 ppm (Table 2).

The dynamics of the ratchet experiments also give considerable information. Not only interspecies or interstrain variation is evaluated. Each replicate evolves as an independent population. Consequently, three independent populations of each strain were also able to be analysed. As a rule, the three replicates of the same strain showed different patterns during the ratchet experiment. Interreplicate differences are easily evidenced at the highest doses of simazine. For example, repliates No. 1 and No. 2 of the *E. huxleyii* strain CCMP373 were able to grow at 0.15 ppm of simazine after 200 d (*c.* 40 generations) and 180 d (*c.* 36 generations), respectively. By contrast, replicate No. 3 of the same strain was unable to adapt at 0.15 ppm simazine. This interstrain variation reveals the effect of chance on the adaptation process.

Finally, a simazine concentration causing 100% growth inhibition in ancestral populations (before the ratchet experiments) is compared between the ancestral populations (before the ratchet experiments) and the derived populations (after the ratchet experiments) (Table 1). Simazine resistance of continental Chlorophyta increases between 90 and 270 times. Resistance of coastal Chlorophyta and continental Cyanobacteria increases *c.* 10 times. Resistance of coastal diatom only increases 4.5 times. Furthermore, resistance of oceanic Haptophyta only increases 1.5 times.

Discussion

Survival and growth of phytoplankton in contaminated environments is an interesting topic from both the genetic and physiological points of view, as phytoplankton is the principal primary producer of aquatic ecosystems (Kirk, 1994; Falkowski & Raven, 1997). In fact, microalgae have been shown to be highly sensitive to the presence of this type of herbicide (Solomon *et al.*, 1996), but herbicide sensibility varies depending on the species tested (Blank *et al.*, 1984; Ma & Chen, 2005).

In spite of species diversity tested here (i.e. Cyanobacteria, Chlorophyta, Bacillariophyta, Haptophyta isolated from continental, coastal and oceanic microalgae), simazine was able to inhibit 100% growth in all ancestral populations. Consequently, the indiscriminate use of herbicides could have extremely negative consequences on phytoplankton. When any toxic substance appears in the environment, the most sensitive organisms are eliminated in favor of the most resistant individuals. This mechanism increases the community tolerance and contributes

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Table 2 Time (*t*) and number of generations (*g*) required to obtain growth under increasing doses of simazine herbicide during the ratchet experiment

Oceanic algae

Strain	Replicate	0–0.15 ppm		0.15–0.45 ppm	
		t (d)	*g*	*t* (d)	*g*
Emiliania huxleyi (*CCMP371*)	*No. 1*	180	36	*U*	–
	No. 2	180	36	*u*	–
	No. 3	160	32	*u*	–
E. huxleyi (*CCMP372*)	*No. 1*	160	32	*u*	–
	No. 2	160	32	*u*	–
	No. 3	180	36	*u*	–
E. huxleyi (*CCMP373*)	*No. 1*	200	40	*u*	–
	No. 2	180	36	*u*	–
	No. 3	*u*	–	–	–
Isochrysis galbana	*No. 1*	160	40	*u*	–
	No. 2	180	45	*u*	–
	No. 3	160	40	*u*	–
Monochrysis lutheri	*No. 1*	180	45	*u*	–
	No. 2	180	45	*u*	–
	No. 3	160	40	*u*	–

Coastal marine algae

Strain	Replicate	0–0.15 ppm		0.15–0.45 ppm		0.45–1.5 ppm		1.5–4.5 ppm	
		t (d)	*g*	*t* (d)	*g*	*t* (d)	*g*	*t* (d)	*g*
Tetraselmis suecica	*No. 1*	20	15	60	45	120	90	*u*	–
	No. 2	20	15	60	45	140	105	*u*	–
	No. 3	20	15	60	45	120	90	*u*	–
Phaeodactylum tricornutum	*No. 1*	40	30	160	120	*u*	–		
	No. 2	40	30	180	135	*u*	–		
	No. 3	40	30	160	120	*u*	–		

Continental waterbody algae and cyanobacteria

Strain	Replicate	0–0.15 ppm		0.15–0.45 ppm		0.45–1.5 ppm		1.5–4.5 ppm		4.5–13.5 ppm		13.5–40.5 ppm		40.5–120.5 ppm	
		t (d)	*g*	*t* (d)	*g*	*t* (d)	*g*	*t* (d)	*g*	*t* (d)	*g*	*t* (d)	*g*	*t* (d)	*g*
Dictyosphaerium chlorelloides	*No. 1*	20	15	40	30	60	45	60	45	80	60	140	105	*u*	–
	No. 2	20	15	40	30	60	45	60	45	100	75	120	90	*u*	–
	No. 3	20	15	40	30	60	45	60	45	80	60	120	90	*u*	–
Scenedesmus intermedius	*No. 1*	20	15	40	30	80	60	100	75	160	120	*u*	–		
	No. 2	20	15	40	30	80	60	100	75	160	120	*u*	–		
	No. 3	20	15	40	30	100	75	120	90	140	105	*u*	–		
Microcystis aeruginosa (Ma3D)	*No. 1*	40	15	160	60	*u*	–								
	No. 2	60	23	160	60	*u*	–								
	No. 3	40	15	140	53	*u*	–								
M. aeruginosa (Ma6D)	*No. 1*	40	15	200	75	*u*	–								
	No. 2	40	15	240	90	*u*	–								
	No. 3	40	15	220	83	*u*	–								
M. aeruginosa (Ma7D)	*No. 1*	60	23	140	53	*u*	–								
	No. 2	60	23	140	53	*u*	–								
	No. 3	40	15	160	60	*u*	–								

u, unable to adapt after 100 d.

to alter its own structure, exerting a differential selection pressure on the community diversity (Blanck & Dahl, 1996).

Besides this ecosystem response, an intraspecific selection pressure occurs and resistant genotypes are selected. Not much is known about the mechanisms allowing adaptation

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of phytoplankton to toxic substances. It is usually assumed that phytoplanktonic organisms can survive in unfavorable environments (i.e. anthropogenic pollution) as a result of physiological acclimation supported by modifications of gene expression (Bradshaw & Hardwick, 1989; Fogg, 2001). Nevertheless, when the values of some environmental factors exceed the physiological limits, the survival is based on adaptive evolution (Sniegowski & Lenski, 1995; Belfiore & Anderson, 2001), which ultimately depends on originating new mutations (Sniegowski, 2005).

The majority of studies have traditionally focused on the degree of tolerance to the herbicide rather than on the capability of adaptation. Ratchet protocol has been designed as a tool to estimate the capability for adaptation in diverse groups, species and strains of phytoplankton. The potential for adaptation to simazine contamination is experimentally estimated maintaining large populations (large enough to maximize occurrence of beneficial mutations). This needs to occur under strong selection pressure, strong enough to favor enrichment of these beneficial mutations in populations. This approach was successfully employed by Reboud *et al.* (2007) with *Chlamydomonas reinhardtii* as an experimental model for research into the evolution of atrazine resistance. A ratchet protocol was also employed to select resistant genotypes of microalgae against a wide range of pollutants that includes pesticides, heavy metals and antibiotics (Orellana *et al.*, 2008).

According to this experimental protocol, the maximal simazine resistance in each experimental population should be achieved by means of genetic adaptation. Marvá *et al.* (2010) demonstrated that green microalgae adapt to low doses of herbicides as a result of physiological acclimation, whereas the survival of these microalgae under high doses of herbicides depends exclusively on new mutations that confer resistance. For example, the same strains of *S. intermedius* and *D. chlorelloides* used in this study were unable to acclimatize physiologically to high doses of simazine. Adaptation of simazine at doses of 3.1 ppm (or higher) was only possible because of the occurrence of rare spontaneous simazine-resistant mutants occurring randomly during replication of organisms before exposure to simazine (Marvá *et al.*, 2010). Consequently, adaptations obtained by our ratchet protocol (i.e. up to 40.5 ppm simazine in *S. intermedius* or 13.5 ppm simazine in *D. chlorelloides*) can only be genetically achieved by new mutations that confer resistance.

Additionally, two distinct results can be found during the ratchet experiment, being interpreted as the independent consequence of two different phenomena. In the first case, if resistant cells arose by physiological acclimation, the number of generations required to obtain growth under increasing doses of simazine should be the same in each replicate of each strain, because every cell is likely to have had the same chance of developing resistance. By contrast, if resistant cells arose by mutation, the number of generations required to obtain growth under increasing doses of simazine should occasionally be different among the replicates of each strain. This is because mutations occur earlier or later in some replicates or perhaps mutational events may not occur in other replicates. Although acclimation and mutation can occur at the same time, the interreplicate variability observed in the ratchet experiment can only be explained if rare spontaneous mutations are the pacemaker of this adaptation.

This study has been extended to a broad group of phytoplankton in order to evaluate their capability for genetic adaptation to a herbicide. This constitutes a novel way of exploring the different possibilities of phytoplankton to adapt genetically to anthropogenic contamination. The ability of a variety of groups of phytoplankton to adapt to simazine was quite variable. Why were some species able to increase their ability to adapt to simazine only 1.5 times, whereas others increased by as much as 270 times?

Taxonomic group, ploidy, growth rate and habitat preference are involved in the ability of different groups of phytoplankton to adapt to simazine (Table 3).

Table 3 Characteristics implicated in the capability of different groups of phytoplankton to adapt to simazine

Species/strain	Adaptation increase	Taxonomic group	Ploidy	Cell division	Habitat preference
Scenedesmus intermedius	270 times	Chlorophyta	Haploid	Rapid	Continental
Dictyosphaerium chlorelloides	90 times	Chlorophyta	Haploid	Rapid	Continental
Tetraselmis suecica	10 times	Chlorophyta	Haploid	Rapid	Coastal
Microcystis aeruginosa (3D)	Nine times	Cyanobacteria	Haploid	Moderate	Continental
M. aeruginosa (6D)	Nine times	Cyanobacteria	Haploid	Moderate	Continental
M. aeruginosa (7D)	Nine times	Cyanobacteria	Haploid	Moderate	Continental
Phaeodactylum tricornutum	4.5 times	Bacillariophyta	Diploid	Rapid	Coastal
Emiliania huxleyi (CCMP373)	Three times	Haptophyta	Haploid	Slow	Oceanic
E. huxleyi (CCMP372)	1.5 times	Haptophyta	Haploid	Slow	Oceanic
E. huxleyi (CCMP371)	1.5 times	Haptophyta	Haploid	Slow	Oceanic
Isochrysis galbana	1.5 times	Haptophyta	Haploid	Moderate	Oceanic
Monochrysis lutheri	1.5 times	Haptophyta	Haploid	Moderate	Oceanic

Cell division: rapid, one doubling every 3–4 d; moderate, one doubling every 3–4 d; slow, one doubling every 5–7 d.

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The Chlorophyta group showed the greatest capacity to adapt to simazine. By contrast, Cyanobacteria did not show as much ability to adaptat. This difference could result from the fact that prokaryotic organisms are more adversely affected by herbicides than eukaryotic species, as has been previously reported (Tomaselli *et al.*, 1987; Fournadzhieva *et al.*, 1995). Several studies concerning adaptation have demonstrated that the Cyanobacteria are usually the most sensitive species to certain pollutants in comparison with eukaryotic microalgae (Bañares-España *et al.*, 2006; López-Rodas *et al.*, 2007). This finding has been attributed to the overlapping respiratory and photosynthetic electron transports in Cyanobacteria, as both pathways share numerous electron transport intermediaries that are the target of the triazine herbicides (Campbell *et al.*, 1998). Bacillariophyta showed a moderate capability for simazine adaptation. Similarly, Haptophyta showed scarce capability to adapt to simazine.

From a classic population genetics point of view, recombination, ploidy and growth rate affect the speed of adaptive evolution (Crow & Kimura, 1970; Klug *et al.*, 2006). Consequently, these details of biology should explain the differential capability for adaptation within the different species. For instance, haploids will respond to selection more quickly than diploids because all nonneutral mutations are expressed immediately. Obviously, the growth rate also affects the speed of adaptive evolution since a greater number of generations during a given time allows for more speedy adaptive evolution. As expected, the species with the greatest rate of adaptation to simazine are haploid populations growing rapidly (Table 3).

With regard to habitat preference, phytoplankton of continental water bodies was the group that showed the greatest ability to adapt to simazine, followed by coastal marine microalgae. It is interesting to note that the increase of intensive agriculture production systems and persistent herbicide usage in agriculture have caused simazine to be common in continental waters as well as in coastal fringes as a result of river discharge. In fact, triazine herbicide-resistant plant populations have been well documented (http://www.weedscience.org/in.asp). Therefore, it is reasonable to assume that triazines may act as a powerful selective agent in continental aquatic environments where these microalgae normally occur and resistance alleles had been selected via the succession of population generations. This same reasoning can be applied in the case of coastal organisms that are likely exposed to this family of herbicides. By contrast, the most sensitive group to simazine was formed by oceanic microalgae. These phytoplankton species occur in habitats without herbicides and can therefore be more vulnerable to sudden exposures to herbicides than other species inhabiting more contaminated regions. Oceanic microalgae showed a reduced capability for simazine adaptation, which may reflect that no prior exposure to the herbicide or other related compound had been experienced by these organisms in the ocean domain. As a consequence, the capability for herbicide adaptation has not advanced during their evolutionary history.

Although there still remain many uncertainties concerning the impact of herbicide contamination on phytoplankton, our study demonstrates that it is possible to obtain a picture of the response of populations to changes in habitat conditions by designing experimental systems that allow the capacity of genetic adaptation of these microorganisms to be evaluated.

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III.III. Warming will affect phytoplankton differently: Evidence through a mechanistic approach

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Warming will affect phytoplankton differently: evidence through a mechanistic approach

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Although the consequences of global warming in aquatic ecosystems are only beginning to be revealed, a key to forecasting the impact on aquatic communities is an understanding of individual species' vulnerability to increased temperature. Despite their microscopic size, phytoplankton support about half of the global primary production, drive essential biogeochemical cycles and represent the basis of the aquatic food web. At present, it is known that phytoplankton are important targets and, consequently, harbingers of climate change in aquatic systems. Therefore, investigating the capacity of phytoplankton to adapt to the predicted warming has become a relevant issue. However, considering the polyphyletic complexity of the phytoplankton community, different responses to increased temperature are expected. We experimentally tested the effects of warming on 12 species of phytoplankton isolated from a variety of environments by using a mechanistic approach able to assess evolutionary adaptation (the so-called ratchet technique). We found different degrees of tolerance to temperature rises and an interspecific capacity for genetic adaptation. The thermal resistance level reached by each species is discussed in relation to their respective original habitats. Our study additionally provides evidence on the most resistant phytoplankton groups in a future warming scenario.

Keywords: phytoplankton; climate change; warming; genetic adaptation; ratchet technique

1. INTRODUCTION

Climate change is now firmly established as a scientific reality, with a variety of emergent challenges for the Earth system in the coming decades. The oceans play a relevant role in modulating the climate system through storage and transport of heat [1], and through the uptake and sequestration of carbon dioxide [2]. According to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [3], the global mean surface air temperature rose by 0.74°C over the last century, while the global mean sea surface temperature increased by 0.67°C in the same period [4]. As the release of excess CO_2 to the atmosphere will continue, the planet and some critical ocean regions may soon be warmer than at any time in the past million years [5,6]. It has been predicted that by the end of the 21st century, the sea surface might experience a temperature augmentation between 1.1°C (low CO_2 emission scenario B1) and 6.4°C (high CO_2 emission scenario A1FI) [7]. Warming will also be experienced by large freshwater bodies, with a rise of 1–7°C in surface water temperatures being predicted under a forecasted doubling of atmospheric CO_2 concentrations [8]. Therefore, there is currently a clear research need to understand the effects of warming on aquatic systems.

The anthropogenically driven temperature rise will disrupt the aquatic environment in many ways. Physical changes are expected, such as modifications in circulation and stratification patterns, which will indirectly have drastic results on biogeochemical cycles of essential elements and biota distribution [9–11]. These alterations will ultimately lead to shifts in food web structure and productivity [12,13]. However, owing to the sensitivity of biological processes to temperature, direct thermal effects on aquatic life forms are also anticipated. From a broad perspective, there are three main response options for organisms facing warming: (i) species may disperse to more hospitable habitats, (ii) phenotypic and physiological plasticity may allow species to tolerate the new conditions, or (iii) species may adapt to the new conditions through genetic change via the process of evolution [14]. In particular, drifting life forms whose spatial distribution is primarily determined by the motion of the water column, such as those integrating the plankton community, rely on the two last mechanisms to cope with the increased temperature, considering the environmental selection forcing. Among this diverse group of organisms, phytoplankton (which are central to biogeochemical and ecological services and play key roles in both regulation of atmospheric CO_2 through photosynthesis and in the maintenance of upper trophic levels) have already been observed to respond to warming.

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Thus, marine phytoplankton biomass and productivity have been shown to decrease in response to warmer sea surface temperatures, although this diminution has been attributed to the indirect effect of the temperature-driven stratification on the isolation of surface waters from cool, nutrient-rich deeper water [9]. The predicted reduction in nutrient supply to the euphotic layer as a result of increased thermal stratification has been also indicated as a potential mechanism altering phytoplankton community composition [10]. A direct effect of warmer temperature on phytoplankton populations has been also described, as a significant increase in the proportion of small-sized species under higher thermal conditions has been evidenced in both freshwater ecosystems [15,16] and in the marine domain [13,16]. In fact, a gradual shift towards smaller primary producers in a warmer ocean has been foreseen, as temperature has been regarded as the main environmental parameter controlling size distribution in phytoplankton assemblages [17]. Therefore, studies addressing the straight effect of temperature on phytoplankton populations in the context of global warming focus mainly on allometric relationships and seldom have they specifically analysed the capacity of individual phytoplankton cells to efficiently adapt to the increased thermal conditions. At present, therefore, it is unclear whether the shifts in phytoplankton species composition can be attributed to a direct metabolic response to changes in temperature or are an indirect effect of variations in light, nutrients and other abiotic or biotic factors associated with modifications in water circulation and climate [18].

Additionally, the polyphyletic complexity of the phytoplankton community does not allow one to establish a general conclusion about the cell mechanisms conferring tolerance to warming, but undoubtedly genetic adaptation will ultimately determine species success and survival in a new thermal scenario. Here, various common phytoplankton species from a number of major groups were investigated in relation to their capacity to cope with a temperature forcing. We analysed, at individual level, the maximum capacity of adaptation to a gradual warming process in species belonging to distinct ecological niches, and discussed their responses in relation to their respective natural habitats. Additionally, this study provides experimental evidence for assessing how phytoplankters might respond and evolve to the envisaged higher temperatures in the near future.

2. MATERIAL AND METHODS

(a) *Organisms and growth conditions*

Sixteen strains of 12 phytoplankton species obtained from the Algal Culture Collection of the Universidad Complutense (Madrid, Spain) and belonging to four different major phytoplankton groups were examined. Thus, phytoplankters isolated from continental freshwater bodies, coastal marine waters, open ocean waters and symbiotic of corals were used. The exact isolation sites along with the natural thermal variation range during the year are indicated in table 1.

The first group, corresponding to phytoplankton from continental waterbodies, comprised one strain of the chlorophyte *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman, one strain of the chlorophyte *Scenedesmus intermedius* Chodat and three strains (*Ma3D*, *Ma6D* and *Ma7D*) of the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann. *Dictyosphaerium chlorelloides* was isolated from a mountain lake from Sierra Nevada (southwest Spain), whereas the rest of organisms were isolated from a pristine lagoon in Doñana National Park (southwest Spain). Phytoplankton from coastal marine waters comprised: the prasinophyte *Tetraselmis suecica* (Kylin) Butcher, isolated from coastal waters of Sardinia (Italy); the free-living dinoflagellate *Prorocentrum triestinum* Schiller from the continental shelf of the gulf of Cadiz (Spain); and the three diatoms (Bacillariophyceae) *Nitzschia closterium* (Ehrenberg) Smith, *Navicula* sp. and *Phaeodactylum tricornutum* Bohlin, which were isolated from coastal waters of Galicia (Spain). Phytoplankton from oceanic waters comprised marine haptophytes—specifically, two strains (*CCMP 371* and *CCMP 372* from the UTEX stock) of *Emiliania huxleyi* (Lohm.) Hay & Mohler originally obtained from the Sargasso Sea—and *Isochrysis galbana* Parke and *Monochrysis lutheri* (Droop) Green, both isolated from the north central Atlantic. Finally, two strains (*CCMP 2433* and *CCMP 2429*) of *Symbiodinium* sp. (Dinophyceae), extracted from corals of the Coral Sea (south Pacific), formed the group of symbiotic phytoplankton.

For each strain, cultures were re-cloned before experiments by separating a single cell in order to avoid the inclusion of genetic variability that might have occurred in the culture by mutations prior to experiments. After isolation, a single cell was asexually grown until around 500 cells were obtained, which were used to create triplicate bottles of 100 cells. Triplicates were grown axenically during 30 days prior to the experiments in ventilated cell-culture flasks covered with a filter cap (Greiner, Bio-One Inc., Longwood, NJ, USA) containing either 20 ml of BG11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany) for experiments with freshwater microalgae and cyanobacteria, or alternatively 20 ml of f/2 medium (Sigma) in the case of their marine counterparts. Flasks were initially placed at 22°C under a continuous photon flux density of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm provided by cool white fluorescent tubes. Cultures were maintained in balanced growth corresponding to mid-log exponential growth by serial transfers of a cell inoculum to fresh medium.

(b) *Experimental design*

Most phytoplankton groups have a great phenotypic plasticity for physiological acclimation to changes in their habitat conditions, which is supported by modifications of gene expression [19]. Nevertheless, when these changes exceed the physiological limits, species survival depends exclusively on adaptive evolution, which is in turn driven by the occurrence of mutations that confer resistance [20]. It is difficult to experimentally estimate the optimum selection pressure that ensures enough events of adaptive mutations is difficult as strong selection pressures drastically reduce population size. This constraint can be overcome by performing experiments that include several levels of the selection agent. Accordingly, an experimental procedure was developed [21]—the so-called ratchet protocol—based on the exposure of large populations of single species to short-term intense selection, which was attained by maintaining a strong selection pressure at a temporal scale up to several months. This technique was subsequently improved [22] through modifications in the original design to maximize the occurrence of mutants and their concomitant selection by applying variable selection pressures. This enhancement was achieved by simply using different

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Table 1. Isolation sites of the strains subjected to the ratchet experiment and annual temperature range in their natural environments.

isolation site	location (lat/long)	species/strain	cell volume (μm^3)	isolation temperature (°C)	annual temperature range (°C)
continental water bodies	037° 006′ N; 006° 028′ W	*Scenedesmus intermedius*	207	21	11–29
	037° 003′ N; 003° 22′ W	*Dictyosphaerium chlorelloides*	78	12	5–27
	037° 005′ N; 006° 029′ W	*Microcystis aeruginosa* (*Ma3D*)	117	23	14–31
	037° 005′ N; 006° 029′ W	*Microcystis aeruginosa* (*Ma6D*)	96	23	14–31
	037° 005′ N; 006° 029′ W	*Microcystis aeruginosa* (*Ma7D*)	70	23	14–31
open ocean	032° 000′ N; 062° 000′ W	*Emiliania huxleyi*[a] (*CCMP 371*)	180	unknown	19–26
	032° 000′ N; 062° 000′ W	*Emiliania huxleyi*[a] (*CCMP 372*)	48	unknown	19–26
	043° 041′ N; 011° 013′ W	*Isochrysis galbana*	30	14	13–19
	043° 007′ N; 010° 046′ W	*Monochrysis lutheri*	70	15	13–19
coastal waters	038° 059′ N; 008° 022′ E	*Tetraselmis suecica*	357	22	13–25
	043° 023′ N; 008° 023′ E	*Phaeodactylum tricornutum*	122	14	13–19
	036° 007′ N; 006° 023′ W	*Prorocentrum triestinum*	670	21	15–24
	036° 007′ N; 006° 001′ W	*Nitzschia closterium*	16	21	15–24
	036° 007′ N; 006° 001′ W	*Navicula* sp.	102	21	15–24
corals	023° 008′ S; 152° 000′ E	*Symbiodinium* sp. (*CCMP 2429*)	1153	unknown	22–28
	023° 008′ S; 152° 000′ E	*Symbiodinium* sp. (*CCMP 2433*)	1022	unknown	22–28

[a]Isolated at 40 m depth.

replicates of each species under the selecting condition, thereby assuring repeatability. The ratchet protocol has been applied in a number of studies to characterize the adaptation of microalgae to extremely hostile habitats [22–27].

Hence, the maximum capacity of phytoplankton to adapt to a warming process can be assessed experimentally through this procedure by analysing the growth of individual species subjected to increasing temperature (as the selecting agent) during many generations. The ratchet protocol permits selection and preservation of the occurrence of both pre-existing and arising mutations that benefit the population and lead to thermal adaptation. Even though this evolutionary approach may be considered an oversimplification of the natural scenario, it still provides a good approximation to the initial stage encountered by an organism in the field when temperature progressively varies.

The procedure followed in this work was aimed at reaching equilibrium between strong selection pressure, by means of ratcheting species to a warmer temperature, and the maintenance of a population size large enough to ensure the occurrence of mutations conferring adaptation. Thus, cultures of individual species were ratcheted only up to a temperature that supported population growth and were exposed to different selection levels. Sixteen independent experiments were conducted (one for each phytoplankton strain). During the early phase, three replicates of control cultures containing growth medium and three replicates of cultures for each temperature value were prepared (see the electronic supplementary material, figure S1). Three initial temperatures were set up at 22°C, 30°C and 35°C. Replicates were grown separately in 5 ml tubes (Sarstedt, Nümbrecht, Germany) inoculated with 3×10^5 cells ml^{-1} of the wild-type population from mid-log exponential growing cultures. This cell concentration was considered large enough to ensure the occurrence of a large final population after applying a temperature rise. In the case of *Symbiodinium* sp. the initial cell density used as inoculum was 10^5 cells ml^{-1}, owing to the lower growth saturation of this species.

All cultures were counted using a particle counter (Beckman Z2, Brea, CA, USA), except for *S. intermedius* cultures, which were counted using a haemocytometer and an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany). Cultures were kept under the selecting temperature value for 15–20 days prior to observation. At this stage, cell concentrations were again counted, and comparison between control and experimental cultures was made. If cell concentration in one of the replicates was similar to or higher than that in control tubes (estimated by mean comparisons of 15 countings using Student's *t*-test), it could be assumed that noticeable growth had been achieved by the population

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under the warmer temperature. The replicate was then ratcheted to the next temperature cycle and subjected to a higher temperature. Replicates that did not reach a cell concentration equivalent to that found in wild-type populations (control cultures) were not transferred (see the electronic supplementary material, figure S1).

In this procedure, each individual tube is considered an independent population. Therefore, if cell density in one of the three replicates belonging to the same initial set was similar to that achieved in control cultures, that particular replicate was ratcheted to the next temperature cycle, regardless of the cell density existing in the other two replicates. This criterion was followed to select different resistance levels that could be attained separately or resistant microalgae likely to occur earlier. In other words, each tube presented a different random chance for particular beneficial mutations, which may arise individually. Both control and ratcheted cultures were again inoculated in this second stage with identical cell concentrations to those used during the first cycle.

A ratchet cycle was concluded when no further cell growth was observed to proceed in a replicate after a period of 100 days. The number of ratchet cycles was then species-dependent as growth was the result of the different adaptation capacity to temperature. The maximum level of resistance of each species was estimated as the highest temperature that allowed the occurrence and growth of a resistant genotype.

Growth rates were calculated before and after the ratchet experiments at the final temperatures of the cycles according to the equation $r = \log_e(N_t/N_0)/t$, where $t = 5d$, and N_0 and N_t are the cell density at the start and at the end of the experiment, respectively. The number of generations during the ratchet experiments was estimated as in [28].

3. RESULTS AND DISCUSSION

(a) *Interpretation of the selection experiments (phenotypic acclimation and genetic adaptation)*

Survival of phytoplankton under temperature increase involves a complex combination of phenotypic acclimation, mutation and selection. Although micro-organisms can survive in unfavourable environments as a result of phenotypic acclimation, which is driven by physiological modifications without genetic changes, when the threshold of an environmental factor exceeds the physiological limits, survival depends exclusively on genetic adaptation, supported by the occurrence of mutations that confer resistance and subsequent selection [20]. Whereas the neo-Darwinian view postulating that adaptation to unfavourable environments occurs by selection on new mutations was widely accepted by the 1940s, many biologists felt that adaptation in microbes (including phytoplankton) might take place through a physiological process [29]. Nevertheless, the unifying neo-Darwinian principles have been experimentally confirmed ever since in numerous studies on phytoplankton adaptation [22–27].

The ratchet protocol has been specifically designed as a tool to estimate the maximum capability for adaptation in phytoplankton, which is obtained by genetic adaptation [29]. Single cells are used to inoculate triplicates of a particular strain, thereby ensuring that initial cultures are clonal (containing only one genotype). Since the propagation of beneficial mutations allows survival at increasing temperatures, the potential for adaptation to a temperature rise is experimentally assessed by maintaining populations large enough to maximize the occurrence of those beneficial mutations under strong selection pressure and to favour their enrichment within populations. Although the ratchet procedure is unable to disentangle the relative contributions of physiological acclimation and genetic adaptation, two distinct results can be found by performing the ratchet experiments, which can be interpreted as the independent consequences of two different phenomena: phenotypic acclimation occurring at physiological level without genetic changes, or genetic adaptation owing to the appearance of new mutations that confer resistance, followed by selection of mutant genotypes [20].

In the first case, if resistant cells arose exclusively by physiological acclimation, the number of generations required to grow under temperature increase should be identical in all replicates of a particular strain, because each individual cell has the same chance of developing resistance. In contrast, if resistant cells arose by mutation, the number of generations required to grow under temperature increase should be different among the replicates of each strain. This effect is due to the fact that mutations appear at different times in the replicates, or perhaps mutational events may not even take place. As indicated in table 2, our results show that the number of generations required to proliferate under a temperature rise differed between triplicates of each strain. Although acclimation and mutation can happen simultaneously, the inter-replicate variability observed in all the strains can only be explained if rare spontaneous mutations are involved in adaptation to the progressive warming. Experimental measures of mutation rates in phytoplankton range from 10^{-5} to 10^{-7} mutations per cell per generation [22–27]. Therefore, the high cell density maintained during the experiments presumably assured the appearance of numerous mutants, which propagated through subsequent generations under the strong selection pressure provided by the ratchet cycles.

Even though each replicate exhibited a different number of generations, triplicates of the same strain invariably reached the same range of temperature tolerance. This repeatability indicates that the ratchet procedure is a good estimator of the maximum capability for adaptation.

Additionally, growth rates of the different strains were measured under the assayed temperatures prior to (ancestral strains) and after (derived strains) the ratchet selection experiments (table 3). If a phytoplankton species was able to survive in an unfavourable temperature only as a result of phenotypic acclimation (physiological non-genetic changes), then the genetically unchanged ancestral genotypes would grow at the same speed after being subjected to a ratchet cycle, and its optimum growth temperature would remain also unmovable. However, growth for genotypes derived from temperature selection obtained after the ratchet experiments showed a very different pattern, in terms of both growth rates and optimum temperatures, than ancestral genotypes prior to the ratchet experiments (table 3). For instance, derived strains of *S. intermedius* were able to grow rapidly at 40°C while ancestral strains were not (table 3). Derived strains of *D. chlorelloides*, *M. aeruginosa*, *I. galbana* and *T. suecica* occurred at 35°C, whereas their respective ancestral strains were unable

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Table 2. Number of generations (g) required to grow under increasing temperature during the ratchet experiment cycles.

isolation site	strain	replicate	22 → 30°C	30 → 35°C	35 → 40°C	40 → 45°C
continental water bodies	*Scenedesmus intermedius*	no. 1	15	30	135	—
		no. 2	15	30	135	—
		no. 3	15	30	150	—
	Dictyosphaerium chlorelloides	no. 1	15	90	—	
		no. 2	15	120	—	
		no. 3	15	90	—	
	Microcystis aeruginosa (*Ma3D*)	no. 1	8	24	—	
		no. 2	8	24	—	
		no. 3	8	16	—	
	Microcystis aeruginosa (*Ma6D*)	no. 1	8	24	—	
		no. 2	8	24	—	
		no. 3	8	32	—	
	Microcystis aeruginosa (*Ma7D*)	no. 1	15	38	—	
		no. 2	15	45	—	
		no. 3	15	45	—	
open ocean	*Emiliania huxleyi* (*CCMP 371*)	no. 1	—			
		no. 2	—			
		no. 3	—			
	Emiliania huxleyi (*CCMP 372*)	no. 1	—			
		no. 2	—			
		no. 3	—			
	Isochrysis galbana	no. 1	10	50	—	
		no. 2	10	50	—	
		no. 3	10	40	—	
	Monochrysis lutheri	no. 1	—			
		no. 2	—			
		no. 3	—			
coastal waters	*Tetraselmis suecica*	no. 1	15	90	—	
		no. 2	15	90	—	
		no. 3	15	120	—	
	Phaeodactylum tricornutum	no. 1	—			
		no. 2	—			
		no. 3	—			
	Prorocentrum triestinum	no. 1	25	—		
		no. 2	25	—		
		no. 3	30	—		
	Nitzschia closterium	no. 1	20	—		
		no. 2	30	—		
		no. 3	20	—		
	Navicula sp.	no. 1	27	—		
		no. 2	34	—		
		no. 3	20	—		
corals	*Symbiodinium* sp. (*CCMP 2429*)	no. 1	65	—		
		no. 2	55	—		
		no. 3	60	—		
	Symbiodinium sp. (*CCMP 2433*)	no. 1	60	—		
		no. 2	70	—		
		no. 3	65	—		

to grow at that temperature (table 3). A similar response was observed in *P. triestinum*, *N. closterium*, *Navicula* sp. and *Symbiodinium* sp. (table 3). These qualitative differences between ancestral strains prior to the ratchet experiments and derived strains after the ratchet experiments corroborate that adaptation was indeed reached by a genetic change (mutation + selection).

In connection with these results, a study aimed at disentangling the effects of physiology, mutation, selection, chance and history in adaptation to temperature increase and eutrophication in marine dinoflagellates has provided evidence of almost no contribution of physiology, chance or history to this process [30]. Also, Gould [31] proposed a theoretical experiment consisting in 'replaying life's tape' to unravel the effects of the aforementioned factors on evolutionary change. His theoretical proposal was empirically addressed by a robust experiment in which, instead of 'replaying life's tape' sequentially, the same objective was achieved by replicating independent isolates propagated simultaneously [32]. Recently, a similar experiment designed to examine the effect of temperature and eutrophication on toxin production in several strains of *M. aeruginosa* has shown that adaptation occurred through new mutations arising during propagation of cultures under the selecting conditions, which displaced the wild-type ancestral genotypes [33].

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Table 3. Growth rates of the different strains under the temperatures assayed prior and after the ratchet experiments (*u*, unable to grow).

	growth rate					
	ancestral strains (before ratchet experiments)			derived strains (after ratchet experiments)		
species	30°C	35°C	40°C	30°C	35°C	40°C
Scenedesmus intermedius	0.52	0.46	*u*	0.46	0.50	0.41
Dictyosphaerium chlorelloides	0.53	*u*		0.53	0.48	*u*
Microcystis aeruginosa (*Ma3D*)	0.28	*u*		0.29	0.22	*u*
Microcystis aeruginosa (*Ma6D*)	0.25	*u*		0.27	0.20	*u*
Microcystis aeruginosa (*Ma7D*)	0.27	*u*		0.30	0.21	*u*
Emiliania huxleyi (*CCMP 371*)	*u*			*u*		
Emiliania huxleyi (*CCMP 372*)	*u*			*u*		
Isochrysis galbana	0.17	*u*		0.18	0.11	*u*
Monochrysis lutheri	*u*			*u*		
Tetraselmis suecica	0.48	*u*		0.51	0.42	*u*
Phaeodactylum tricornutum	*u*			*u*		
Prorocentrum triestinum	*u*			0.16	*u*	
Nitzschia closterium	*u*			0.35	*u*	
Navicula sp.	*u*			0.21	*u*	
Symbiodinium sp. (*CCMP 2429*)	*u*			0.17	*u*	
Symbiodinium sp. (*CCMP 2433*)	*u*			0.19	*u*	

(b) *Specific growth responses to temperature*

When the usual threshold of an environmental condition changes, the most sensitive organisms are excluded and the most resistant individuals become favoured. This mechanism increases community tolerance and contributes to alter its own structure, exerting a differential selection pressure on community diversity [19].

In addition to this ecosystem response, an intraspecific selection pressure occurs and resistant genotypes are selected. The growth responses obtained here when cells were successively exposed to increased temperatures seem to be coherent with this pattern. By raising the temperature in consecutive cycles, the ratchet technique resulted in a relatively rapid evolution of phytoplankters, although each species showed a particular level of thermal resistance (table 2), which was achieved by genetic adaptation through the appearance of mutants that displayed different growth requirements than their respective parental genotypes (table 3).

Results reveal that, on the grounds of temperature alone, there are clear interspecific differences in phytoplankton survival of a gradual warming process (table 2). Phytoplankton species isolated from continental water bodies characterized by a wide range of temperatures throughout the year (tables 1 and 2) were found to occur at a temperature of 35°C, and even at the highest temperature assayed (as for *S. intermedius*, which resisted up to 40°C). During the first cycle, in which ancestral wild-type strains were ratcheted to 30°C, there were no differences in the number of generations (*g*) between replicates of the same species. The number of generations required to reach the same cell density as in control cultures was 15 in all cases except for *M. aeruginosa* (*Ma3D* and *Ma6D*), which took eight generations (table 2). More differences between replicates of the same species were observed during the second ratchet cycle (from 30°C to 35°C), with *D. chlorelloides* being the organism that needed a higher number of generations to achieve the cell density found in ancestral populations. However, in all cases, *g* rose in relation to that obtained in the first cycle (table 2). In *S. intermedius*, the only species able to efficiently adapt to 40°C after applying a third ratchet cycle, *g* clearly increased in comparison to that needed to adapt to lower temperatures. This organism did not show appreciable growth after ratcheting the temperature to 45°C.

On the other hand, phytoplankton species of open ocean waters exhibited a limited resistance to increased temperatures, and with the exception of *I. galbana*, neither the two strains of *E. huxleyi* nor *M. lutheri* were tolerant of the rise from 22°C to 30°C (table 2). When *I. galbana* was exposed to 35°C, the generations required to achieve the growth of wild-type population notably increased with respect to the first ratchet cycle, being similar among the three replicates (table 2). A third cycle did not lead to growth in this species. The response to warming observed in coral symbionts differed, as the two strains of *Symbiodinium* were able to adapt to 30°C. Nevertheless, it is worth noting that all replicates experienced the highest numbers of generations (approx. 60) to reach cell densities equivalent to those attained by control experiments in relation to all the species that overcame the first cycle (table 2). A second ratchet cycle, increasing the temperature from 30°C to 35°C, did not result in adaptation in this species. Growth of the five phytoplankton species isolated from coastal waters responded differently to warming, and while *T. suecica* was able to resist temperatures up to 35°C, the diatom *P. tricornutum* exclusively proliferated at 22°C (table 2). On the other hand, the other two diatom species *N. closterium* and *Navicula* sp., as well as *P. triestinum*, were tolerant to the rise from 22°C to 30°C. During the first ratchet cycle, *Tetraselmis* exhibited a lower number of generations to grow than the rest of the coastal species, although when this organism was taken from 30°C to 35°C, *g* was found to be six- or eightfold higher (depending on the replicate) than that required during the first cycle.

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As indicated above, the design of the ratchet experiments provides additional information, since not only is inter-species or inter-strain variation evaluated, but also the capacity of each replicate to evolve as an independent population. Therefore, the inter-strain variations observed are evidence of the effect of chance on the adaptation process.

The global biogeography of phytoplankton is determined by local environmental factors that select for species based on their optimal growth potential. Among these factors, temperature plays a fundamental role, and in fact the influence of warming on the regulation of phytoplankton dynamics has been reported in aquatic systems including lakes [34] and the open ocean [9,10,17]. Also, the direct influence of temperature on growth rates of microalgae has been broadly evidenced in marine [35] and freshwater species [36]. Consequently, temperature has been always regarded as an effective indicator for phytoplankton distribution in nature. Field observations in continental water bodies and marine systems suggest shifts in phytoplankton occurrence in response to increased water temperature [10–13], although such changes in species succession in the natural habitat are not consistent for all functional groups [18,37], and the general trend described indicates that, in nature, warming favours smaller size classes [16–18]. The majority of studies have been focused on the degree of tolerance to temperature or its effect at ecosystem level rather than on the individual capacity of adaptation. However, both approaches can be combined in order to explain some of the responses already described. Thus, a new model [11] predicts that high temperatures would favour the proliferation of cyanobacteria blooms directly through increased growth rates. The approach proposed by these authors explains the development of the harmful cyanobacterium *Microcystis* during hot summers in eutrophic lakes [11]. The adaptative response of this genus (table 2) is in agreement with the field observations and their modelled tendency, as the three strains of *M. aeruginosa* were able to thrive at temperatures up to 35°C, even though chance affected the adaptation time since both the generations required for optimum growth (table 2) and growth rates (table 3) varied under the new conditions. The fact that this species can genetically adapt to a temperature rise has serious ecological implications in future scenarios, as dense surface blooms of toxic cyanobacteria may lead to mass mortalities of fish and birds, and may represent a serious health threat for cattle, pets and humans [38]. From a classic population genetics point of view, recombination and ploidy must also be taken into account to analyse the speed of adaptive evolution [20]. It is known that haploids respond to selection faster than diploids because non-neutral mutations are quickly expressed. The ploidy of *Microcystis* can therefore be directly related to its ability for mutants to rapidly gain traction in a new environment. Overall, the species with the greatest level of adaptation to warming were haploid populations (table 2).

The rest of the phytoplankton isolated from continental water bodies were also characterized by a great tolerance to high temperatures. In particular, *Scenedesmus*, which even proliferated at 40°C (table 2), possesses a considerable and rapid ability to adjust its cellular physiology, metabolism and growth to relatively large increases in growth temperature [39,40]. Our results indicate that its phenotypic plasticity is based on an elevated capacity for genetic adaptation. Similarly, the cosmopolitan *Dyctiosphaerium* can tolerate a wide range of temperatures, which can be also explained by its rapid genetic adaptation, as already reported in *D. chlorelloides* [41] and corroborated by the ratchet protocol (table 2). This experimental procedure also showed that S*ymbiodinium* occurred up to 30°C, with higher temperatures resulting in a collapse of derived populations (table 2). This finding is in agreement with the fact that photosynthesis in symbiotic dinoflagellates is impaired at temperatures above 30°C and completely ceases at 34–36°C [42]. As warming and photoinhibition are the primary triggers of coral bleaching, many efforts are being devoted to identify the consequences of the heat stress on the symbiotic association between coral and *Symbiodinium*. Previous studies have indicated that the functional response of the symbiosis is indeed temperature-dependent. However, symbiosis breakdown varies between algae of different clades, with some clades being more susceptible to elevated temperatures than others [43,44]. A plausible explanation for distinct heat tolerances may stem from a different thermal sensitivity of the repair of photodamaged photosynthetic machinery among clades. Thus, while severe photoinhibition was observed at temperatures exceeding 32°C in some cells grown at 25–34°C, other more thermally tolerant individuals seemed unaffected [45,46]. In a field transplant study, corals that changed their dominant symbiont type to clade D, a well known thermally tolerant variety of *Symbiodinium*, increased their thermotolerance by 1–1.5°C [47]. In addition, high temperatures have been observed to correlate with the distribution of *Symbiodinium* type in corals, with the symbiont type changing (and possibly conferring thermotolerance) during natural bleaching events. The two strains assayed here could be well integrated into the group of clades more vulnerable to heat stress but with a relatively medium tolerance to a temperature rise in relation to the rest of the species analysed (table 2). The free-living red-tide-forming dinoflagellate *P. triestinum* was also able to adapt to 30°C, coinciding with its habitat preference, as this species is normally found to proliferate during mid-summer in coastal areas characterized by temperatures as high as 30°C [48,49]. Moreover, it appears that dinoflagellates prefer warmer temperatures, which may be a reflection of mixotrophy and the influence of temperature on heterotrophic metabolism or flagellar motility [18]. In contrast, the coccolithophorids considered here displayed a considerable sensitivity to high temperatures, as growth was not measured above 22°C (table 2). Coccolithophores are, on average, most successful (in terms of diversity and proportion of the total phytoplankton community) in warm, oligotrophic, low-latitude waters [50]. It has been suggested that temperature itself plays a direct role in the success of the group, although there is little hard data in support of this contention. *Emiliania huxleyi* thrives even in the relatively cold waters of the north Atlantic south of Iceland, the Patagonian Shelf and the Barents Sea [51]. It seems that, on geological time scales, coccolithophores have adapted to a long-term decrease of atmospheric CO_2 and cooling ocean temperatures by decreasing their coccolith and cell size.

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Therefore, if this organism has evolutionarily become adapted to oligotrophic habitats in moderate-temperature waters, it is not surprising that its growth was found to be completely inhibited above 22°C. In fact, cultured *E. huxleyi* has been shown to grow at a temperature range of 10–25°C [52], which is indicative of its preference for warm temperatures and in agreement with our observations. This result contrasts with that found in *I. galbana*, the other haptophyte considered (table 2), which is a non-calcifying species. The distinct temperature tolerance found within this group can be explained on the basis that calcification is strongly influenced by temperature [52,53] and also by taking into account that *I. galbana* occurs in more tropical latitudes than *E. huxleyi* does.

Regarding species isolated from coastal waters, *Tetraselmis* is typical of mid-latitude coastal areas characterized by large annual temperature variations, and, along with *Isochrysis*, has been extensively used in aquaculture worldwide. This circumstance could have favoured the appearance of resistance alleles that have been selected throughout the succession of derived population. Surprisingly, *P. tricornutum* was unable to grow above 22°C, and although this species can cope with higher temperatures, it is also true that its proliferation at 30°C has never been reported. This response contrasts with that exhibited by the other two diatoms, of the genera *Navicula* and *Nitzschia*, which can be considered generalists such as are known to thrive in continental margins ([54] and references therein).

(c) *Implications*

The majority of phytoplankters analysed here display a genetic adaptation to warming that seems to be mainly related to the thermal conditions of the natural habitat they have been selected for at a geological time scale. The species that were more flexible and well adapted to the temperature range assayed in this study were those normally encountered in temperate aquatic systems characterized by thermal fluctuations all year round. This finding confirms the accepted view that colder-water communities often lack the genetic redundancy required to withstand an environmental change such as warming. Several studies have highlighted that small organisms are more able to tolerate increased temperature and it has been proposed that global warming will benefit small-sized phytoplankton taxa in aquatic ecosystems [16,17]. An environmental selection towards smaller primary producers would have profound implications for biogeochemical cycles [17,18] and food web structure [13,18]. Nevertheless, our study does not support this notion at an evolutionary level, as the genetic capacity to cope with a temperature rise did not seem to be related to cell size, considering the wide spectrum of cell volumes displayed by the chosen species (table 1). In fact, this trend was also observed intraspecifically, as strains of the same species with different cell volume (e.g. *Microcystis*, *Emiliania* and *Symbiodinium*) evolved in a similar manner under warmer scenarios (tables 1 and 2). These results suggest that the observed shift towards a dominance of small-celled phytoplankton communities [16,17] would have its primary origin in a temperature-driven environmental process, such as nutrient supply or preferential grazing owing to the impact of warming on zooplankton, rather than in a direct thermal effect on phytoplankton metabolism. At present, phytoplankton evolution in a warmer world remains unpredictable, and it is clear that future research must address how the expected temperature rise will alter phytoplankton evolutionary succession. A broad range of work is therefore required to enhance our predictive capabilities. Although the mechanistic technique used in this study constitutes an oversimplification of reality, the evolutionary approach by which the expected temperature rise has been simulated can be considered a novel way to explore the maximum capacity for genetic adaptation to the future thermal scenario in phytoplankton key groups. Our data show that a wide variety of interspecific responses are expected to occur based on the different capabilities of phytoplankters to genetically adapt to a warmer ocean. Such capacity will undoubtedly cause shifts in the composition of the phytoplankton community, as well as replacement of impaired individuals by others that are more resistant; or low-latitude marine species could even colonize higher latitudes as the global sea surface temperature becomes warmer. Although an absolute scenario cannot be envisaged at this point, it is certain that genetics will ultimately determine which species will survive to the environmental forcing.

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CAPITULO IV

CONTRIBUCIÓN DE LAS DISTINTAS FUERZAS EN EL CAMBIO EVOLUTIVO

"*Nothing in biology makes sense except in the light of evolution*"

(Th. Dobzhansky, 1973)

Las consecuencias del calentamiento global sobre los distintos sistemas terrestres, y en concreto, sobre los sistemas acuáticos han sido y son ampliamente estudiadas. Distintas predicciones revelan efectos en la fisiología de los organismos (fotosíntesis, respiración, tasa de crecimiento) así como cambios en la distribución de especies y productividad (Hughes 2000; Hays 2005). Una de las consecuencias importantes, como resultado del aumento de la temperatura terrestre, es la proliferación de blooms de fitoplancton tóxico, en concreto cianobacterias, en ecosistemas continentales (Jöhnk 2008). La proliferación de estos blooms está asimismo asociada a fenómenos de eutrofización (Anderson 2002) por un aporte masivo de nutrientes derivados fundamentalmente de excedentes antropogénicos. Las consecuencias ecológicas del aumento de temperatura o nutrientes sobre el desarrollo de blooms de fitoplancton han sido ampliamente estudiadas pero no existen demasiados trabajos examinando el desarrollo desde un punto de vista evolutivo.

En el presente capítulo se estudia la evolución de la cianobacteria *Microcystis aeruginosa* en un escenario de aumento de la temperatura y aporte de nutrientes. Para ello se pretende determinar la contribución de tres componentes fundamentales del cambio evolutivo: la adaptación, el azar y la contingencia histórica en la evolución de dos parámetros de interés en esta especie: la tasa de crecimiento y la toxicidad.

IV. Evolutionary changes in growth rate and toxin production in the cyanobacterium Microcystis aeruginosa under a scenario of eutrophication and temperature increase

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Evolutionary Changes in Growth Rate and Toxin Production in the Cyanobacterium *Microcystis aeruginosa* Under a Scenario of Eutrophication and Temperature Increase

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Abstract Toxic blooms of the cyanobacterium *Microcystis aeruginosa* affect humans and animals in inland water systems worldwide, and it has been hypothesized that the development of these blooms will increase under the future scenario of global change, considering eutrophication and temperature increase as two important consequences. The importance of genetic adaptation, chance and history on evolution of growth rate, and toxin production of *M. aeruginosa* was studied under these new conditions. The experiment followed the idea of "replaying life's tape" by means of the simultaneous propagation of 15 independent isolates of three *M. aeruginosa* strains, which were grown under doubled nutrient concentration and temperature during c. 87 generations. Adaptation by new mutations that resulted in the enhancement of growth rate arose during propagation of derived cultures under the new environmental conditions was the main component of evolution; however, chance also contributed in a lesser extension to evolution of growth rate. Mutations were selected, displacing the wild-type ancestral genotypes. In contrast, the effect of selection on mutations affecting microcystin production was neutral. Chance and history were the pacemakers in evolution of toxin production. Although this study might be considered an oversimplification of the reality, it suggest that a future scenario of global change might lead to an increase in *M. aeruginosa* bloom frequency, but no predictions about the frequency of toxicity can be made.

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Introduction

The toxin-producing cyanobacterium *Microcystis aeruginosa* (Kützing) Kützing frequently occurs in dense blooms in reservoirs and lakes, and its toxins (microcystins) are considered to be the most prevalent cyanobacterial toxins in freshwater ecosystems [50]. In fact, *M. aeruginosa* is the most important cause of toxic cyanobacterial blooms affecting humans and animals in inland water systems worldwide [9, 13, 30, 45, 50, 51]. Moreover, long-term exposure to low levels of microcystins in drinking water can be a risk factor for liver and colorectal cancer [38]. In addition, toxic cyanobacterial blooms pose a challenge for wildlife conservation because they are the cause of repeated mass mortalities of water birds and fishes [1, 10, 37].

Nowadays, humankind is considered the greatest evolution cause [42]. Civilization's new practices have derived in a global change causing severe alterations in ecosystems worldwide [58]. In particular, inland freshwater systems are being transformed rapidly by eutrophication and temperature increase [11]. These changes will probably intensify in the next decades following both the expected increase in global temperatures and inputs of anthropogenic nitrogen. Different works have reported a warming of at least 5°C above today's mean by 2,100 [29] and double anthropogenic nitrogen inputs by 2,050 [54]. Phytoplankton can be considered a likely target to experience this environmental

forcing since nutrient availability and temperature are among the main conditions that decide competitive advantage and regulate phytoplankton species distribution [18]. Depending on which species or groups are affected and in what manner, variations have the potential to alter inland freshwater ecosystems, in particular, if toxic cyanobacteria species are favored. Recently, it has been suggested that the frequency and duration of cyanobacterial blooms are increasing due to summer heat waves [31, 40, 41]. The adaptation to new conditions linked to global change can be achieved by two different kinds of mechanisms: ecophysiological responses or genetic adaptation. Ecophysiological responses (acclimation) are the result of modification of the genes expression already present in populations while genetic adaptation counts with the selection of new genetic variants. The ecophysiological reactions of cyanobacteria under environmental change have been previously addressed [19]. In contrast, as far as we know, no experiments have been carried out to analyze evolutionary responses of cyanobacteria to environmental forcing. However, evolutionary change includes much more forces than genetic adaptation [26]. At least other two factors also contribute to evolution: chance and historical contingency. The effects of chance are usually due to genetic drift events and random mutations without value for the organisms [16, 32, 53]; the final consequence is that alleles that neither improve nor decrease adaptation are maintained in populations. Historical contingency can become important if certain genetic changes of adaptive value in the past constrain or promote evolutionary outcomes [24]. To disentangle the effects of adaptation, chance and history on evolutionary change, Gould [25] proposed a theoretical experiment, which consisted of "replaying life's tape" to test the repeatability of evolution and thereby to evaluate their respective roles; the experiment was envisioned to demonstrate the processes involved in macroevolutionary events. Obviously, an experiment such as that envisioned by Gould cannot be performed and as Lenski and Travisano stated [34]: "the limitations reflect our lack of access to better machines for time travel". However, this theoretical proposal can be empirically addressed in microevolution by the robust experiment of Travisano et al. [55] in which, instead of "replaying life's tape" sequentially (where each tape record is a replication of the experiment, each of them separated in time), one can achieve the same objective by replicating independent isolates propagated simultaneously.

At the starting point, identical isolates (replicates) from a single ancestral genotype are established and the initial mean value of a specific phenotypic trait (in our case, growth rate and microcystin production) is measured for each of them. This value is expected to be identical among isolates, within statistical limits of measurement error, at the beginning of the experiment. After a period of time, the value of each trait is measured again for each isolate. Differences between the initial and final mean values are explained as a result of the effect of adaptation, chance, or history [55]. Thus, a significant change in the mean value in relation to that of the ancestral isolate means that the trait has been a target of natural selection or that it is correlated with some other trait that has been selected. On the other hand, a significant increase in its variance represents the occurrence of divergence among the evolved isolates (the specific trait has not been a target for adaptation but reflects the effects of random mutations or drift or their interactions with other evolutionary processes). Other alternatives are the occurrence of both adaptation and chance, or on the other hand, the occurrence of neither of them (see [55]). To test the effect of history, it is necessary to carry out a similar experiment using different ancestral genotypes [55].

The experiment of Travisano et al. [55] was designed for bacterial populations, and with the appropriate modifications, has been recently addressed with digital organisms [57] and in a marine dinoflagellate species [22]. Certainly, this experimental evolutionary study can be carried out in any microorganism that can be grown asexually and easily manipulated during many generations.

The aim of this study was to investigate the evolutionary response of bloom-forming, toxic and non-toxic strains of *M. aeruginosa* to new environmental conditions (simulating a global change scenario, i.e., high levels of nitrate and elevated temperature). For this purpose, the relative importance of different evolutionary components (genetic adaptation, chance, and historical contingency) on the change of two relevant phenotypic traits of *M. aeruginosa* was analyzed. Likewise, we tried to check whether possible adaptation could be explained as acclimation or as the selection of new genetic variants. One of the selected traits was the growth rate, considered as the main component of fitness and, in consequence, an important trait in evolutionary theory [21]. The other selected trait was the toxin production, crucial for its ecological and management implications [1, 45]. We expect that growth rate is likely to exhibit directional selection (adaptation) and convergent evolution. However, knowing the weak genetic correlation of toxin production with fitness [35], as well as the unclear toxin function [28, 44, 48, 49, 59], we should expect a major influence of chance events and the retention of ancestral differences. Even though this study might be considered an oversimplification of the reality, the evolutionary ecological approach followed here constitutes a novel way to explore, throughout a rigorous experimental model, the evolutionary response of toxic cyanobacteria to anthropogenic-induced changes in environmental conditions.

Methods

Experimental Organism and Growth Conditions

Experiments were carried out using three *M. aeruginosa* strains (Ma3D, Ma6D, and Ma7D) from the Algal Culture Collection, Genetics Laboratory, Facultad de Veterinaria, Universidad Complutense (Madrid, Spain). Strains were collected from a pristine lagoon in Doñana National Park (SW Spain) with 0.032 M of nitrate and 22°C and were maintained in culture collection 3 years prior to the experiments under 0.035 M of nitrate and 22°C conditions (BG-11 medium. Sigma, Aldrich Chemie, Taufkirchen, Germany). Data on isolation of the strains were described by Carrillo et al. [12], and information about their ecophysiological characterization, genetic variability, and the genetic bases of resistance against several biocides of the strains are included in [3, 4, 15, 35, 36, 47]. In particular, Ma3D and Ma6D are non-toxic strains, while the strain Ma7D is toxic [12].

Cultures were grown axenically in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany) at 22°C under a continuous irradiance of 60 μmol m^{-2} s^{-1} over the 400–700 nm waveband supplied by daylight fluorescent tubes. Cells were maintained axenically in mid-log exponential growth by serial transfers of an inoculum to fresh medium once every 2 weeks. Only cultures without bacteria were employed.

Experimental Design

A detailed description of the experimental design is provided in reference [22]. Just before the experiment, the three strains were re-cloned from a single cell, thus assuring their genetic homogeneity at the starting point. These newly established cultures were grown to high densities in environmental conditions similar to those used to maintain the cultures started from stocks from the Algal Culture Collection (BG-11 medium, 22°C, and continuous irradiance of 60 μmol m^{-2} s^{-1} over the 400–700 nm waveband). Cultures were then used to found 15 independent isolates of each strain, and their acclimated maximal growth rate (a proxy of the Malthusian parameter of fitness) and their toxin concentration (amount of microcystin-LR equivalent per cell) were measured (see Fig. 1). These

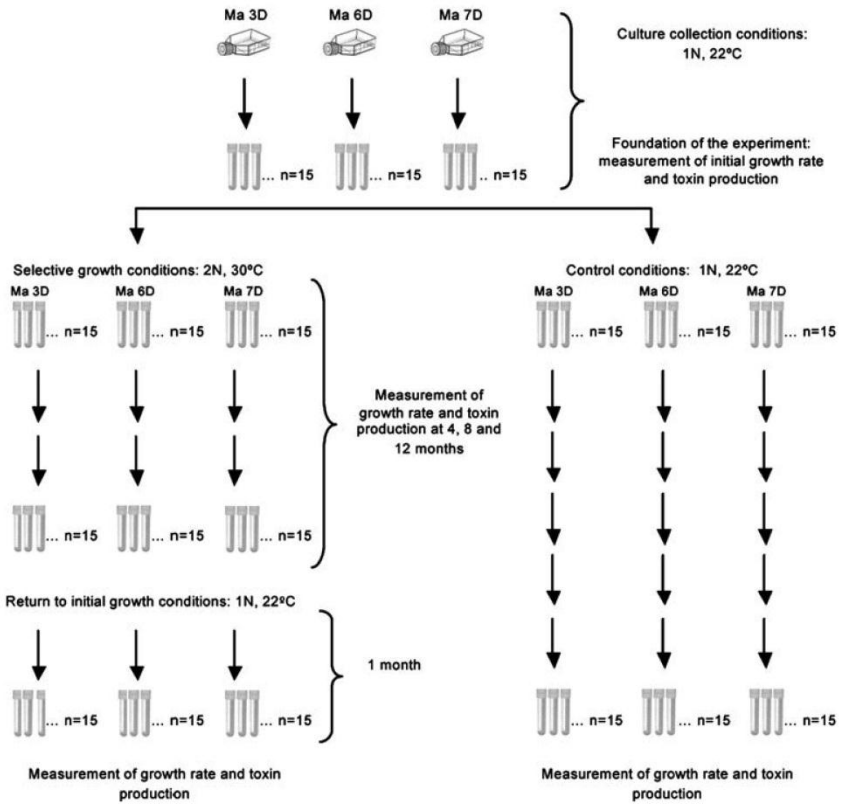

Figure 1 Schematic representation of the experimental design. Fifteen isolates for control and 15 isolates for new selective growth conditions were used for each of the three *Microcystis aeruginosa* strains. Cultures were maintained through serial transfer into fresh new medium for 1 year (corresponding to c. 87 generations): control conditions in BG-11 culture medium (1N) and 22°C and selective growth conditions in BG-11 with double concentration of nitrate (2N) and 30°C. Growth rate and toxin production were estimated at the beginning, every 4 months, and at the end of the experiment. In order to discriminate adaptive change due to physiological adaptation (acclimation) or by genetic adaptation, cultures were transferred to the initial culture conditions for 1 month (c. 7 generations), and growth rate and toxin production were measured again after this period

isolates were transferred to new culture conditions, which were selected in order to mimic a global change scenario with increased nitrate levels and elevated temperature. Therefore, the cultures were transferred to BG-11 medium with double concentration of nitrate (0.07 M of nitrate) and 30°C. The remaining culture conditions (i.e., photon irradiance and photoperiod) were maintained as previously described.

Experimental cultures were serially propagated by 32-fold dilution into fresh medium every 21 days, which allowed about five generations of binary division before the further addition of fresh medium ($5=\mathrm{Log}_2 32$). Cultures were propagated for 1 year (corresponding to c. 87 generations). Growth rate and toxin production were checked every 4 months. Finally, averaged growth rate and toxin concentration of the evolved cells were estimated and compared with those of the ancestral ones.

The effects of adaptation, chance, and history on evolutionary change of growth rate of *M. aeruginosa* were estimated by using the values measured at the start of the experiment and after 12 months under the new experimental conditions (see Fig. 1). In particular, the effects of adaptation were defined by changes in the mean value, and 95% confidence limits were calculated by using the t distribution. The effects of chance and history were estimated by means of a two-level (3 strains and 15 isolates within each strain, with 3 replicates of the growth rate measurement per isolate) nested ANOVA. Thus, the contribution of the chance component corresponds to the variance measured among isolates within the same strain, whereas the history component was estimated by the variance among strains. The homogeneity of variances was checked with the F test and the normality of the data by the Kolmogorov–Smirnov test. The square root of the variance component for chance and history was reported in order to use units that were comparable to the mean change due to adaptation. Approximate 95% asymmetrical confidence limits were calculated for the variance components. In the case of toxin production, homogeneity of variances was not achieved even by transforming data. Furthermore, toxin production was just expressed in one of the strains (7D). Therefore, the non-parametrical Mann–Whitney U test was used to compare ancestral and derived values from the toxic strain 7D. All the statistical procedures were performed in accordance with reference [52].

Acclimation vs. Genetic Adaptation

In order to test if adaptation is supported by acclimation or by selection of the new genetic variants arising by mutations, the cells cultured in the selective growth conditions (c. 87 generations at double nitrate concentration, 30°C) were then newly transferred to the initial culture conditions (original nitrate concentration, 22°C) for 1 month (c. 7 generations). As control, parallel cultures were maintained in the initial growth conditions (original nitrate concentration, 22°C) for the duration of the experiment (i.e., 12+1 months). The growth rate and toxin production of each experimental replicate was measured and compared with that found in the controls. Changes in mean values were calculated using ANOVA. Seven generations under the initial conditions are considered enough to check if adaptation process is assured by means of acclimation or genetic mechanisms (reviewed by Cooper [14]).

It is hypothesized that similar growth rate and toxin production values mean that adaptation to selective growth conditions was achieved by acclimation because the return to the initial growth conditions yielded similar results. On the contrary, different growth rate values and toxin production mean that the genetic adaptation occurred during the time when cultures were submitted to selective growth conditions.

Measurement of Growth Rate and Toxicity

Acclimated maximal growth rate (an estimator of Malthusian parameter of fitness) was estimated under r selection conditions in exponentially growing cultures according to [16] as:

$$\text{Growth rate} = \mathrm{Log}_e(N_t/N_0)/t,$$

where N_t and N_0 are the cell number at time t=4 and 0 days, respectively. For this purpose, the values of N_0 and N_t were determined at 3 and 7 days after the transference of cells to fresh medium. Cell numbers in experiments and controls were counted (in triplicate) using a Beckman (Brea, CA, USA) Z2 particle counter. In addition, two independent observers also made cell counts by haemocytometer (Double Neubauer ruling, Fortuna W.G. Co., Germany) to check the accuracy of the particle counter.

The toxin production of the different replicates of each strain was measured using a microcystin-specific enzyme-linked immunoabsorbent assay (ELISA) test (EnviroGard Microcystin Quantitube Test Kit, Strategic Diagnostic, Inc.), according to the manufacturer's recommendations. This kit is a quantitative test for the detection of microcystins residues in water. Prior to the measurements, cell concentration in each replicate was assessed at the late logarithmic growth phase, when toxicity is highest [8], and an aliquot was kept. Then, the cells of each aliquot were broken by freezing and sonication. The insoluble cell debris was removed by centrifugation (15,000 g−force×10 min). The toxin concentration was expressed as nanograms of MCYST-LR equivalent per cell.

Results

Growth Rate

Mean growth rate of the three strains increased during the first 8 months (c. 58 generations). Afterwards, growth rate apparently remained constant (Fig. 2a). Figure 2b shows derived values for growth rate after c. 87 generations in double nitrate concentration and 30°C vs. ancestral values in single nitrate concentration and 22°C in 15 isolates of the Ma3D, Ma6D, and Ma7D strains.

Adaptation was absent (by design) at the start of the experimental selective period. The initial effect of chance on growth rate of *M. aeruginosa* (estimated by the variance among isolates within strains) was absent (F=1.48; df=42 and 90; P>0.5). On the contrary, the initial historical contingency contribution on the growth rate (estimated by the variance among strains) was significant (F=18.94; df=2 and 42; P<0.001) (Fig. 3). After 12 months under the experimental selective period, approx. two thirds of the evolutionary change of the growth rate of *M. aeruginosa* was explained by adaptation (t=13.24; df=133; P<0.001) while approx. one third of evolution was due to chance (F=4.32; df=42 and 90; P<0.001) (Fig. 3). The footprint of the historical contingency on the growth rate of the different strains of *M. aeruginosa* was non-significant (F=0.33; df=2 and 42; P>0.5) at the end of the experiment (Fig. 3).

Mean growth rate value was found to be invariant in control cultures during c. 87 generations (i.e., single nitrate dose and 22°C during the entire length of the experiment) and similar to the growth rate value measured at the outset of the experiments (F=2.76; df=1 and 28; P>0.1). However, the cells derived after c. 87 generations in double nitrate doses and 30°C, newly transferred and cultured c. 7 generations to the original conditions (single nitrate dose and 22°C) for 1 month showed significant higher growth rate values (F=5.30; df=1 and 28; P<0.05) than control cultures after c. 87+7 generations.

Toxin Production

The toxin production in all the isolates of Ma3D and Ma6D remained below the detection limit during c. 87 generations in selective conditions (double nitrate concentration, 30°C), but the isolates from Ma7D showed abundant microcystin production (Fig. 4a). A significant difference between the initial and final 7D values was observed (c. 87 generations; U=52; n_1, n_2=15, 15; P<0.05). Figure 4b shows derived values for mean toxin production after c. 87 generations in double nitrate concentration and 30°C versus ancestral values in single nitrate concentration and 22°C in 15 isolates of Ma7D.

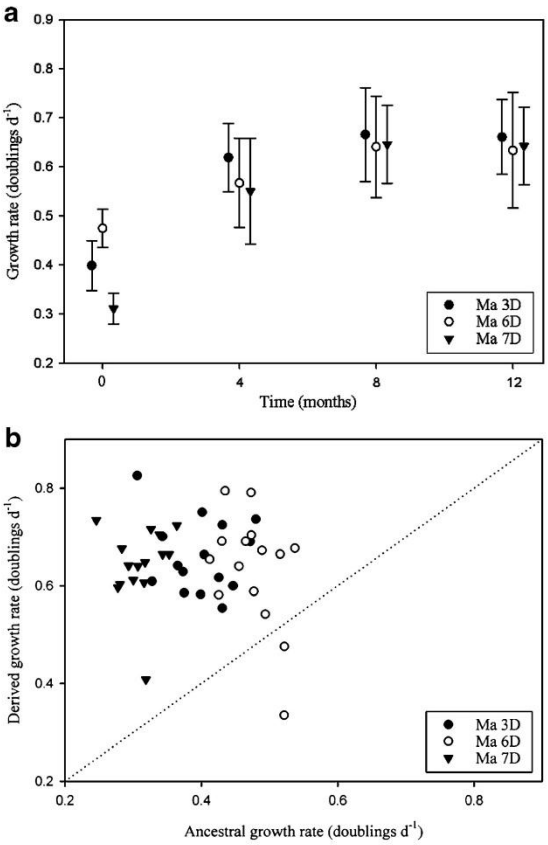

Figure 2 Evolution of growth rate during c. 87 generations in selective conditions (double nitrate concentration and 30°C) (**a**) and derived versus ancestral values for mean growth rate (**b**) in 15 isolates of three strains of *Microcystis aeruginosa*—Ma3D (*close circles*), Ma6D (*open circles*), and Ma7D (*close triangles*). Symbols in **b** correspond to the overall mean (CV<1.5%) of three replicates of the growth rate measurement per isolate and per strain. Isocline (*dotted line* in **b**) represents the score location if no changes take place

A significant difference between the initial variance (at the start of the experiment) and the final variance (after c. 87 generations in the new environmental conditions of doubling nitrate and 8°C temperature increase) was observed in Ma7D (F=9.31; df=1 and 28; P<0.001), suggesting that chance made a significant contribution to evolution of toxin production. Although the effect of history in evolution of toxin production cannot be checked statistically by ANOVA, it could be evidenced because: (1) the non-toxic strains Ma3D and Ma6D remained non-toxic during c. 87 generations in the new environmental conditions of doubling nitrate and 8°C temperature increase; and (2) the toxin-producing Ma7D strain maintained a high level of toxicity during c. 87 generations at the new environmental conditions.

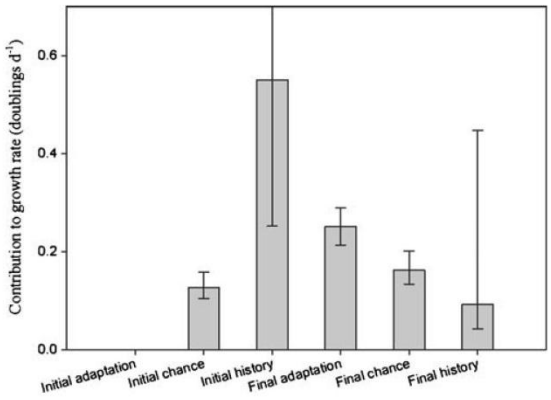

Figure 3 Relative contributions of adaptation, chance, and history to growth rate at the start and at the end of the experiment. *Error bars* represent 95% confidence limits

The Ma7D cells derived after c. 87 generations in double nitrate doses and 30°C, newly transferred and cultured during c. 7 generations to the original conditions (single nitrate dose and 22°C), showed significantly lower toxin production values than control cultures of Ma7D after c. 87+7 generations under the original conditions (U=20; n_1, n_2=15, 15; P<0.001). However, the mean toxin production value also varied in control cultures during c. 87+7 generations under single nitrate dose and 22°C during the entire length of the experiment (U=34; n_1, n_2=30, 30; P<0.001). This is further evidence to demonstrate that chance played an important role in the evolution of this trait.

Discussion

The understanding of the biological response to global change will be one of the main tasks of evolutionary biologists in the coming decade [5]. Investigating the capacity of phytoplankton to respond to the predicted changes in environmental conditions has become a key issue in order to further understand future repercussions on the functioning of aquatic ecosystems [18]. In this context, the importance of estimating the adaptive potential of toxic cyanobacteria to environmental forcing is obvious, but not much is known about the mechanisms implicated in this process [31, 40]. Assuming the complexity of possible responses that may arise to cope with the changing environmental conditions, the experimental approach followed here constitutes a novel way to explore the roles played by physiological adaptation (acclimation), genetic adaptation (selection of favored mutants), chance (genetic drift and neutral mutations), and historical contingency in the adaptation of toxic cyanobacteria under an experimental scenario of increasing temperature and nitrate. Evidently, this study might be considered an oversimplification of the reality, but reductionist approaches have propitiated the development of modern biology [60].

Ever since Fisher [21], the importance of fitness on evolution is well known. For that reason, the evolution of growth rate (the main estimator of fitness) of *M. aeruginosa* after a rapid environmental change was one of the considered parameters in the current study. Genetic adaptation was the predominant component of growth rate evolution. Since asexually growing clonal cultures were used, the evolutionary changes are due to new mutations, which occurred during propagation of derived cultures under the new environmental conditions. Numerous mutations should have arisen in each isolate (due to the huge

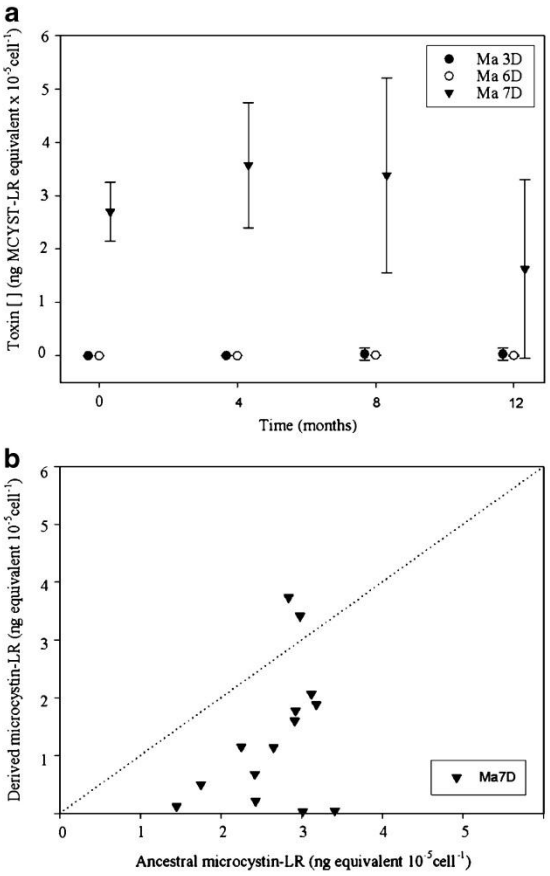

Figure 4 Evolution of toxin production during c.87 generations in selective conditions (double nitrate concentration and 30°C) in 15 isolates of three strains of *Microcystis aeruginosa*—Ma3D (*close circles*), Ma6D (*open circles*), and Ma7D (*close triangles*) (**a**) and derived versus ancestral values for mean toxin production in 15 isolates of the Ma7D strain (**b**). Isocline (*dotted line* in **b**) represents the score location if no changes take place

number of cells growing in each culture). Some mutations increased growth rate and were selected, displacing the wild-type genotypes [22]. Mutations decreasing growth rate were eliminated by natural selection. Increased nutrient concentrations and temperature not only induced selection for increased growth rate, but this selective effect seemed to be strong enough to constrain the evolution of all the experimental isolates, whose growth rates converged at the end of the experiment, as was demonstrated by the low contribution of chance and history. Finally, chance contributed in a lesser extension than adaptation to evolution of growth rate of *M. aeruginosa*, suggesting that some neutral mutations which affected growth rate could arose during the experiment.

Previous studies using viruses [7, 17], bacteria [5, 33] parasitic protozoa [43], a dinoflagellate [22], and even digital organisms ([57]; in this case, award rate, a variable closely related to average fitness, was used) have also reported the occurrence of fitness convergence in sets of isolates propagated under identical, novel environmental conditions. As long as a similar derived growth rate value was achieved, not only within each set of evolving isolates, but also between the strains, it seems that environmental conditions determined the outcome for growth rate to a large extent. Consequently, adaptation was the main component of growth rate evolution and the footprint of history was eliminated, as may occur for traits that are subject to strong selection pressure [35].

It has been observed that the majority of the evolutionary change in a long-term experiment with bacteria occurred during the first generations, followed by evolutionary stasis [34]. Thus, it could be assumed that the c. 87 generations of *M. aeruginosa* along the experiment was enough to detect evolutionary change. In particular, the greater change in growth rate occurred during the first 58 generations after the population was placed in the experimental environment, followed by a period of quasi-stasis. Moreover, some previous studies demonstrated very rapid adaptive evolution of *M. aeruginosa* to lethal selective agents [23, 36].

On the other hand, toxin production by cyanobacteria is crucial from a practical point of view (i.e., management of water supply reservoirs and wildlife conservation). For this reason, evolution of toxicity after a rapid change in environmental conditions was also considered as an important parameter to study. Chance was the predominant component in the evolution of microcystin production in *M. aeruginosa*. Taking into account the constant experimental conditions (in contrast with field conditions) as well as the high number of cells in the experimental cultures, we should consider that the probability of drift events was low and most of the chance effects could be attributed to random mutations. The evolutionary changes are due to new mutations arising during propagation of the derived cultures under the new environmental conditions. Since each culture has a huge number of cells, numerous mutations should have arisen during cell growth in each isolate. Some mutations increased toxin production whereas other mutations decreased toxin production, suggesting that the effect of selection for microcystin production in derived population (i.e., under double nitrate and +8°C) was neutral. Thus, neither toxin-increased nor toxin-decreased mutants have selective advantage. Moreover, it could be supposed that wild-type ancestral genotypes have no selective advantage. Consequently, natural selection was not strong enough to constrain the evolution of the experimental isolates. So, the effect of history was maintained during evolution of microcystin production under new environmental conditions.

There are diverse hypothesis about the role of microcystins in natural populations of cyanobacteria. It has been hypothesized that the microcystins are involved in basic metabolism, protective effects against predators and competitive organisms, alleophatic interactions and others [28, 44, 48, 49, 56, 59]. However, biological functions of microcystin remain controversial. For instance, cyanobacteria are among the most ancient organisms on the Earth [27], and it has been proposed that microcystin synthesis is an ancient process, developed long before the evolution of eukaryotic photoautotrophs [2]. Microcystin synthetase genes were in the past more widely present in cyanobacteria than today [46]. This apparently loss of microcystin synthetase genes during evolution indicates that selection pressure on microcystin production has decreased. It could be possible that toxin production is purely fortuitous, and "non-toxic" strains could have similar structurally related but non-toxic compounds serving the same physiological function [39]. Recently, it has been showed that under environmental conditions that favor cyanobacteria growth, the cost of microcystin production prevails over its benefits, and consequently, toxin production is lowered [6].

An evolutionary approach to this controversy could be useful. It is well established in evolutionary theory that traits that are strongly correlated with fitness evolve by adaptation (selection of favorable mutations); whereas traits that are not (or are very weakly) correlated with fitness evolve by chance [32, 55]. The effect of history is preserved only in traits that are less important to evolution [25, 26, 55]. As it has been previously hypothesized, our study demonstrates that growth rate, which is strongly correlated with fitness, evolved mainly by adaptation, although chance events also contributed to some extent. In contrast, microcystin production was not (r=−0.091, df=14, P>0.05 in this study) correlated with growth rate (the main component of fitness); thus, toxin production evolution was modulated by chance and history, but not by adaptation. Similarly, previous papers showed a very high phenotypic:

genetic variance ratio of microcystin production in experimental populations of *M. aeruginosa* [35]. Quantitative genetic theory proves that only the traits that are not (or are scarcely) important in evolution have high phenotypic: genetic variance ratio [reviewed in 20]. Consequently, toxin production of cyanobacteria had no adaptive value (it seems to be purely fortuitous).

Finally, these results could lead to tentatively hypothesize that the increase of temperature and eutrophication might increase cyanobacteria blooms via growth rate increase. In contrast, since chance is the pacemaker of evolution of toxin production, predictions of the future of toxicity may be impossible. However, the mere fact of increasing cyanobacterial blooms could aggravate problems in water reservoirs due to the unpredictability of the toxin evolution.

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CAPITULO V

EFECTO DE LA DE LA DISPONIBILIDAD DE NUTRIENTES BAJO DISTINTOS ESCENARIOS DE CO_2

"Human beings are now carrying out a large-scale geophysical experiment of a kind that could not have happened in the past nor be reproduced in the future".

(Roger Revelle. Scripps Institute of Oceanography, 1950)

Uno de los retos de actualidad en la comunidad científica es el entender y analizar los efectos de la acidificación oceánica debido al aumento de las emisiones antropogénicas de CO_2. Cambios pequeños en el pH del agua podrían dar lugar a impactos severos en los ecosistemas marinos. Pero dado que el océano es un sistema dinámico y que otras variables ambientales son modificadas simultáneamente, la predicción de las consecuencias globales es tarea complicada.

En el presente capítulo se presenta un trabajo desarrollado para explorar el efecto combinado de la alteración de dos variables ambientales: el aumento de dióxido de carbono atmosférico y disponibilidad de nutrientes, sobre la fisiología del cocolitofórido *Emiliania huxleyi*. Por un lado se investigaron las repercusiones sobre la incorporación del carbono tanto durante el proceso fotosintético como del de calcificación. Asimismo, se profundizó en el estudio del efecto combinado de estas dos variables sobre la asimilación de nutrientes esenciales para el metabolismo como son el nitrógeno y el fósforo. Para ello, se analizó la modificación en la actividad de las dos enzimas implicadas: nitrato reductasa y fosfatasa alcalina, junto a otra serie de enzimas, en la asimilación del nitrato y fosfato respectivamente. Este trabajo pretende aportar un mayor conocimiento para el entendimiento de la fisiología, dinámica de "blooms" y papel futuro de *Emiliania huxleyi* en el ciclo del carbono.

V. The effects of nitrate and phosphate availability on *Emiliania huxleyi* physiology under different CO_2 scenarios

Referencia:

Rouco M, Branson O, Lebrato M, Iglesias-Rodríguez D. Effect of nitrate and phosphate availability on *Emiliania huxleyi* physiology under different CO_2 scenarios (in preparation).

VII. DISCUSIÓN

"Somewhere, something incredible is waiting to be known"

(Carl Sagan)

"Si existe una vida en cualquier otro lugar del Universo, basada en procesos químicos alternativos, entonces podría florecer en los ambientes más extraños y sería difícil considerar que haya planetas donde no floreciera alguna forma de vida"

(Paul Daves. Físico)

Los registros fósiles señalan una antigüedad de las cianobacterias de más de 3000 millones de años (Schopf y Packer 1987). Asimismo, eucariotas fotosintéticos evolucionaron hace más de 1.5 billones de años en los océanos del proterozoico (Falkowski 2004). A diferencia de otros organismos que surgieron a lo largo de la larga vida de la Tierra y cuyo destino final fue la extinción, las cianobacterias y eucariotas fotosintéticos están presentes en los medios acuáticos y terrestres actuales, contribuyendo en gran medida al mantenimiento de la vida en la Tierra tal y como hoy la conocemos. Esto significa que estos organismos tuvieron que sobrevivir extinciones masivas y fenómenos de cambio global extremos en los que la supervivencia de un sinfín de organismos no fue posible. De hecho, actualmente, existen un gran número de ambientes naturales extremos donde la presencia de microalgas y cianobacterias es notable, a pesar de sus características extremas (Albertano *et al.* 1995; Steinberg *et al.* 1998; Rai y Gaur 2001; Baos *et al.* 2002; Pedrozo *et al.* 2001; Donachie *et al.* 2002; Baffico *et al.* 2004; Flores-Moya *et al.* 2005; Beamud 2007; Costas *et al.* 2007, Costas *et al.* 2008; López-Rodas *et al.* 2008c). Pero, ¿Cómo consiguieron estos organismos sobrevivir hasta el presente periodo geológico?, ¿qué mecanismos permitieron su adaptación asegurando su supervivencia y proliferación? De la misma manera nos planteamos ¿cómo consiguen adaptarse ante el actual cambio global que está aconteciendo? y, ¿conseguirán adaptarse ante cambios venideros que acontecen de forma rápida debido fundamentalmente a la participación humana?

A pesar de que esos organismos son excelente modelos para el estudio de la evolución (Elena y Lenski 2003), los estudios de evolución experimental en

fitoplancton no han sido muy abordados (Sniegowski 2005). Por otro lado, la tolerancia de estos organismos a cambios ambientales bruscos es relevante desde un punto de vista ecológico, considerando su papel en el funcionamiento de los ecosistemas acuáticos. A lo largo de esta tesis se han planteado diversos trabajos para abordar estas cuestiones utilizando distintos diseños experimentales.

Las aguas tóxicas procedentes de ambientes extremos pueden considerarse laboratorios naturales en los que se presenta un gradiente de toxicidad natural. De tal manera, facilita entender el proceso adaptativo que permite la supervivencia de microalgas y cianobacterias mesófilas en ambientes con toxicidad diversa y poder comprender la diferente diversidad de organismos fitoplanctónicos presentes. Cuando la clorofita *Dictyosphaerium chlorelloides* y la cianobacteria *Microcystis aeruginosa* fueron expuestas a las aguas de distintos ambientes extremos (aguas del Arroyo de Aguas Agrias; Vulcano y Río Agrio Argentino) se observó un efecto tóxico elevado, como puede verse por la inhibición total del crecimiento y alta inhibición de la eficiencia fotosintética. Las aguas procedentes de estos ambientes presentan grandes cantidades de metales pesados y un pH bajo (Pedrozo *et al.* 2001; Cortese *et al.* 1986; Sánchez-Rodas *et al.* 2006), limitando la supervivencia de los fotosintetizadores mesófilos. Sin embargo, a pesar de su gran toxicidad, estas aguas sustentan una comunidad fitoplanctónica más o menos diversa habitando por encima de los límites de su tolerancia fisiológica. Por ello, estos organismos debieron desarrollar distintos mecanismos adaptativos para asegurar su supervivencia.

El desarrollo del análisis de fluctuación (López-Rodas *et al.* 2001) nos permitió discernir entre dos hipótesis: si la adaptación de los individuos tuvo lugar tras entrar en contacto con el agua extrema, en respuesta directa a ésta o si la adaptación de la población tuvo lugar gracias a la selección de mutantes resistentes que surgieron al azar previamente al contacto con el agua tóxica. Los resultados del análisis confirman que el mecanismo concreto que lleva a la adaptación de los organismos depende en gran medida del grado de toxicidad del agente selectivo y la capacidad de resistencia y adaptación del organismo en concreto. De ahí se explica igualmente la diversidad de

especies encontrada en estos ambientes (Tabla I). Por ello, en las aguas menos tóxicas tanto *Dictyosphaerium chlorelloides* como *Microcystis aeruginosa* pudieron adaptarse mediante procedimientos fisiológicos o de aclimatación, correspondiéndose con los ambientes en los que fue observada la mayor diversidad de organismos (Tabla I). Estos organismos fueron capaces de modificar la expresión de sus genes para permitir el fenómeno adaptativo. Sin embargo, la adaptación a ambientes muy tóxicos o fruto de un efecto catastrófico (como es el caso de las aguas del Río Agrio Inferior cercano al lago, aguas de Vulcano o las aguas Agrias del sur de España) sólo pudo asegurarse a través de una adaptación genética, mediante la selección de individuos resistentes que aparecieron de forma espontánea. En estos casos, la diversidad de especies observada en estas aguas fue más reducida, no observándose la presencia de cianobacterias en ningún caso (Tabla I). En última instancia, si la toxicidad de un ambiente natural es extremadamente elevada la adaptación no puede ocurrir por ningún mecanismo, como ocurrió en las aguas del Río Agrio superior o en el caso de *Microcystis aeruginosa* para la mayoría de las aguas, excepto para las menos ácidas. Este mismo fenómeno ha sido igualmente observado en aguas termales de toxicidad extrema (Costas et al 2008; tabla II). Así, *Microcystis aeruginosa* fue incapaz de adaptarse a aguas a pH menor de 4.1. Resultados similares fueron observados en trabajos previos en aguas del Río Tinto (Costas *et al.* 2007; tabla I) o fuentes geotermales (Costas *et al.* 2008; tabla I). Este hecho puede estar asociado tanto a la ausencia de transportadores ATPasa específicos en estos organismos a diferencia de los organismos eucariotas (Amaral Zettler *et al.* 2003) como a la localización periférica del aparato fotosintético en lugar de en el cloroplasto, como sucede en algas eucariotas (Brock 1978). Las cianobacterias presentan una capacidad excepcional para resistir temperaturas extremas, sequía y altas salinidades (Rai y Gaur 2001). Sin embargo, la existencia de cianobacterias no se ha reportado en ambientes extremadamente ácidos (Brock 1973, 1978; Knoll y Bauld 1989, Albertano *et al.* 1995, revisado por Gimmler 2001; Nixdorf *et al.* 2001) sugiriendo un pH límite de 4.8 para su proliferación (mayor que en el caso de nuestras aguas de estudio) (Brock 1973). No obstante, diversos estudios describen la presencia de algas filamentosas en aguas a pH 2.9 en unos lagos ácidos en Alemania (Steinberg *et al.* 1998). Por ello, este postulado

podría ser válido para cianobacterias unicelulares pero no para especies filamentosas. En todo caso, se correspondería con nuestros hallazgos, puesto que *Microcystis aeruginosa* es un organismo unicelular.

La adaptación a ambientes extremos cuya toxicidad supera los límites de tolerancia fisiológica de los microorganismos parece difícil. De hecho, se asume que algas mesófilas poseen una capacidad limitada para habitar ambientes con pH tan extremos. Por ello, desde un punto de vista clásico se podría considerar que la adaptación genética ante tales condiciones es un proceso gradual. No obstante, trabajos previos han demostrado que la mayor parte de las especies eucariotas que habitan aguas tan extremas como las del Río Tinto al Sur de España (Amaral Zettler *et al.* 2002) se encuentran más estrechamente relacionadas con especies neutrófilas que con acidófilas. Por ello, cabe suponer, que el proceso de adaptación de dichas especies no pudo producirse de forma gradual sino que tuvo que ser el resultado de un proceso rápido, en concreto, a través de la presencia previa de organismos resistentes cuya resistencia fue adquirida por mutaciones aleatorias, antes de entrar en contacto con el ambiente selectivo. De esta manera, estos organismos podrían ser seleccionados rápidamente ante la presencia del agente tóxico. Los trabajos presentados en esta tesis así como otros trabajos en la misma línea (ver tabla I) demuestran tal asunción .Pero los organismos fitoplanctónicos no sólo soportan presiones selectivas derivadas de fenómenos naturales. En los últimos años la liberación masiva al medio acuático de residuos procedentes de la actividad humana compromete en gran medida su supervivencia. Por ello, el estudio de su capacidad adaptativa a estos agentes exógenos resulta interesante desde un punto de vista tanto evolutivo como ecológico. En esta tesis se estudió la capacidad adaptativa de dos clorofitas, *Dictyosphaerium chlorelloides* y *Scenedesmus intermedius* a distintos contaminantes antropogénicos: el formaldehido, el antibiótico cloranfenicol, el metal pesado cromo, y los herbicidas simazina y diquat. Todos los contaminantes, aplicados a dosis elevadas, ejercieron una fuerte presión selectiva en las poblaciones reduciendo la densidad celular de los cultivos. Sin embargo, tras un periodo de tiempo algunas de las poblaciones lograron recuperarse sugiriendo la presencia de células resistentes en esos cultivos. El análisis de fluctuación demostró que en todos los

casos, la resistencia surgió por medio de mutaciones espontáneas que se produjeron al azar durante la replicación de los organismos antes de entrar en contacto con el tóxico. Estos resultados coinciden con trabajos anteriores que consideraron igualmente la adaptación de cianobacterias y microalgas ante diversos contaminantes (Tabla II).

Mediante el uso del análisis de fluctuación es difícil observar mutaciones post-selectivas si la tasa para ese tipo de mutaciones es $< 10^{-8}$. Asimismo, procesos fisiológicos podrían ocurrir simultáneamente a mecanismos genéticos. Pero el análisis de fluctuación no es un procedimiento cuantitativo (Cairns *et al.* 1998; Fox 1998) y, por ello, en el caso de que varios de estos procedimientos tuvieran lugar al mismo tiempo, no se podría discernir entre cada uno de ellos.

Por el contrario, el análisis de fluctuación sí permite la determinación de la tasa de mutación (Luria y Delbrück 1943). La evolución adaptativa depende en última instancia del origen de nuevas mutaciones (Sniegowski 2005), ya que la mutación es la fuente última de variación genética requerida para el proceso de adaptación (Sniegowski *et al.* 2000). Por tanto, el estudio de las distintas tasas de mutación de los diferentes organismos considerados da idea de su éxito evolutivo y capacidad adaptativa (Klug y Cummings 1997). Las tablas I y II de este capítulo resumen las tasas de mutación de una variedad de especies de fitoplancton ante distintos agentes selectivos, tanto aquéllos estudiados en esta tesis como otros analizados en estudios previos. Agrupando todos estos resultados con el fin de determinar la frecuencia en que se producen estas mutaciones se puede concluir que: (1) las tasas de mutación en organismos fitoplanctónicos varían entre 10^{-5} y 10^{-7} mutaciones por célula y por generación. Un último estudio aportó una tasa del orden de 10^{-8} en respuesta de *Dictyosphaerium chlorelloides* al TBT (López- Rodas *et al.* 2010a); (2) la tasa de mutación varía entre especies, cepas y sustancia tóxica, y no se aprecia una fuerte asociación con respecto al origen de cada una, aunque hay que considerar que todas las cepas provenían de ambientes parecidos; (3) las tasas de mutación podrían variar en función de los genes analizados dentro de la misma especie y cepa dependiendo de la mayor o menor

probabilidad de aparición de la mutación que confiere la resistencia, según la diana de mutación; (4) en general, las tasas de mutación espontánea en microalgas y cianobacterias son de dos a tres órdenes de magnitud mayores (según el caso) que aquellas de bacterias (Klug y Cummins 1997) y mayores que las medidas para la resistencia a antibióticos en otras especies de microalgas como *Chlamydomonas sp.* (Sager 1962, 1977). Esto podría explicarse debido a que las poblaciones bacterianas son mucho mayores que las de microalgas y cianobacterias y, consecuentemente una menor tasa de mutación es suficiente para asegurar la supervivencia. Esto mismo ocurre en organismos que presentan reproducción sexual como *Chlamydomonas sp*. Las poblaciones de organismos fitoplanctónicos estudiados en este trabajo y otros (revisado en tablas I y II) presentan densidades celulares menores que las poblaciones bacterianas y no poseen reproducción sexual. Por ello, puede argüirse que necesiten de mayores tasas de mutación para asegurar su supervivencia.

Pero la mutación que confiere resistencia tiene un coste asociado (Coustau *et al.* 2000). La adaptación a un nuevo ambiente tiene un efecto negativo pleiotrópico sobre la *fitness* en un ambiente no selectivo. Es el denominado "coste de adaptación" (Purrington 2000; Strauss *et al.* 2002) que previene incluso la fijación de nuevos alelos beneficiosos (Tian *et al.* 2003) manteniendo así el polimorfismo genético dentro de las poblaciones (Antonovics y Thrall 1994). El estudio del "coste de adaptación" de los alelos resistentes en una población resulta interesante a la hora de predecir la dinámica evolutiva de una resistencia concreta (Neve *et al.* 2003). El "coste de adaptación" asociado al gen de resistencia ha sido ampliamente estudiado para distintos organismos y sustancias tóxicas (Groeters *et al.* 1994; Chevillon *et al.* 1995, Gassmann y Futyuma 2005; Heidel *et al.* 2004; Purrington 2000). Así, puede estar asociado a distintos mecanismos: (1) que la mutación que confiere la resistencia ante el agente determinado comprometa asimismo determinadas funciones metabólicas del organismo, (2) que produzca una compensación entre el crecimiento y funciones de defensa y (3) que las interacciones ecológicas de estos organismos se vean alteradas (revisado por Vila-Aiub *et al.* 2009).

Debido al "coste de adaptación", los organismos resistentes son eliminados por selección por competición frente a organismos sensibles. Trabajos previos han demostrado que tras pocas semanas de competencia entre organismos sensibles y resistentes en ambientes no selectivos, los primeros acaban desplazando a los segundos completamente (Costas *et al.* 2001; García-Villada *et al.* 2004). Entonces, ¿cómo puede ser que dichos organismos permanezcan en las poblaciones naturales en ausencia del agente selectivo? En poblaciones pequeñas, los mutantes serán conducidos a extinción debido a este proceso de selección de los organismos con mayor *fitness* o eficacia biológica. Sin embargo, en las poblaciones naturales, en las que su tamaño es lo suficientemente grande como para asegurar una variabilidad genética elevada, la aparición del alelo resistente es recurrente. En cada generación aparecen nuevos alelos mutantes aunque la mayoría desaparecerán antes o después por selección natural o azar (Spiess 1989). Pero siempre existe un porcentaje de mutantes resistentes que no son eliminados y se mantienen en la población. El número medio de mutantes se determina como el balance entre la tasa de mutación y la tasa de eliminación selectiva (Kimura y Maruyama 1966): $\mu (1-q) = qs$. En consecuencia, $q= \mu/ \mu + s$, donde μ corresponde a la tasa de mutación, q es la frecuencia del alelo mutante y s el coeficiente de selección.

La frecuencia del alelo mutante (q) ha sido determinada en los distintos trabajos de esta tesis y comparado con estudios previos (tabla I y II), observándose que varía entre especies y agentes selectivos. Sin embargo, la frecuencia del alelo mutante (q) para los resistentes a contaminantes antropogénicos parecen ser del orden de 3 y hasta 4 veces mayores que los de resistentes a ambientes extremos (exceptuando las aguas de la Hedionda) (Tabla I y II), lo cual sugiere que las poblaciones podrían responder de forma más rápida a la adaptación frente a la exposición a este tipo de contaminantes.

Cuando el producto del tamaño de una población (N) por su tasa de mutación por locus (μ) es lo suficientemente grande (>1), la variabilidad genética presentada es suficientemente elevada como para permitir la adaptación rápida de la población ante una fuerte presión selectiva (Wahl y Krakauer 2000). Debido a la elevada densidad

celular en poblaciones microalgales, así como la fracción de células sensibles frente a resistentes presentada por estas especies, los niveles de mutación espontánea obtenidos (que otorgan el paso de sensibilidad a resistencia), parecen ser suficiente para garantizar la supervivencia de los organismos en ambientes extremos. De esta manera, podría proponerse que las microalgas presentes en la actualidad en las aguas de los distintos ambientes naturales extremos estudiados, podrían ser las descendientes de aquellos mutantes que aparecieron fortuitamente en algún momento en su historia evolutiva antes de que estuvieran expuestos a estas condiciones extremas (Sniegowski 2005). Asimismo, ante un escenario de cambio global causado por actividades humanas, como puede ser el vertido de herbicidas o antibióticos al medio acuático, la presencia de células previamente resistentes asegurará el desarrollo y mantenimiento de estas poblaciones. No obstante, el coste asociado a la mutación (*fitness* reducida y densidad celular menor) conducirá a la reducción de parámetros ecológicos importantes, tales como la producción primaria o la biomasa microalgal (López-Rodas *et al.* 2001).

Estos trabajos han contribuido a evidenciar la posibilidad de la evolución adaptativa en estos organismos. El fitoplancton es capaz de adaptarse a cambios ambientales extremos en función de la intensidad de la presión selectiva, lo que ayuda a comprender la diversidad encontrada en ambientes extremos, así como la adaptación a medios contaminados. Asimismo, se ha observado una capacidad de adaptación distinta de la clorofitas *Dictyosphaerium chlorelloides* y *Scenedesmus intermedius* y la cianobacteria *Microcystis aeruginosa* ante determinados ambientes. El fitoplancton se agrupa en 11 filas distintos (Margulis y Schwartz 1982) entre los que se encuentran especies con características fisiológicas muy diversas, especies procariotas y eucariotas, con distinta ploidía y tipo de reproducción. Por ello, es de esperar que la respuesta ante un cambio ambiental se desarrolle de forma distinta para cada una de ellas. Ante un aumento elevado y rápido de la presión selectiva como el que se augura ¿continuará la coexistencia espacio-temporal de especies como sugiere la paradoja del plancton (Hutchinson 1961)? O de lo contrario, las poblaciones serán gobernadas por un número escaso de organismos, como sugiere el principio de exclusión competitiva (Hardin 1960). Hutchinson ya propuso que la explicación para la diversidad encontrada en una

masa de agua tanto dulce como marina podía ser debida a que las comunidades fitoplanctónicas no se encuentran en equilibrio debido a fluctuaciones estacionales, climatológicas o hidrológicas, considerando que las condiciones homogéneas propuestas por el principio de exclusión-competitiva es difícil que ocurran: *"Twenty years ago in a Naturalists' Symposium, I put (Hutchinson 1941) forward the idea that the diversity of the phytoplankton was explicable primarily by a permanent failure to achieve equilibrium as the relevant external conditions changed" (*Hutchinson, 1961). Esta hipótesis ha sido ampliamente estudiada y aceptada por infinidad de trabajos (Scheffer *et al.* 2003, Huisman *et al.* 2001, Roy y Chattophadhyay 2007). Sin embargo, resultados de esta tesis muestran que la diversidad de organismos fitoplanctónicos se ve mermada en ambientes extremos (Tabla I). Los trabajos presentados en esta tesis investigan la capacidad diferencial del fitoplancton, así como la máxima capacidad de estos organismos para adaptarse a distintas presiones selectivas. Para ello, como ya se ha puesto de manifiesto, se utilizaron especies pertenecientes a una diversidad de grupos fitoplanctónicos (Chlorophyta, Cyanophyta, Bacillariophyta, Haptophyta y Dinoflagellata) y que presentaban distinto hábitat de preferencia. Se incluyeron organismos de aguas continentales, costeras, de océano abierto y organismos simbióticos de los corales. De esta forma, se pretendía investigar, si la capacidad diferencial de adaptación era debida únicamente al grupo taxonómico o dependía así mismo de su hábitat de preferencia, que condicionaba, por tanto, su historia evolutiva. Todos los organismos estudiados fueron haploides, a excepción de una especie diploide, la diatomea *Phaeodactylum tricornutum*. Entre las presiones selectivas se incluyeron el aumento de temperatura asociado en el medio natural al calentamiento global y la exposición a dos contaminantes antropogénicos, la simazina y el sulfato de cobre, comúnmente detectados en ambientes acuáticos.

Los resultados de los experimentos de Ratchet demostraron una capacidad de adaptación diferente dependiendo tanto de especies y grupos taxonómicos como de su lugar de aislamiento (Tabla III). Los organismos pertenecientes al fila Chlorophyta fueron los organismos capaces de adaptarse a los mayores niveles de selección en todos

los casos, seguido de Cyanophyta, Bacillariophyta y Haptophyta en orden descendiente. Distintos trabajos ya han demostrado que la sensibilidad a herbicidas varía según especies (de Filippis y Pallaghy 1994; Ma 2005). Así, las cianobacterias han mostrado una capacidad destacada para resistir temperaturas extremas, sequía y altas concentraciones de sales (Fogg 2001). Entre las algas eucariotas, las clorofitas han presentado una resistencia extraordinaria ante distintos ambientes extremos (Fogg 2001). De hecho, tan solo cianobacterias y clorofitas han sido capaces de colonizar hábitats terrestres (Fogg 1969). Respecto a su hábitat, los organismos aislados en aguas continentales exhibieron la mayor capacidad de adaptación en todos los casos. La presencia recurrente de un tóxico en el ambiente ejerce un efecto en las comunidades excluyendo los individuos y especies sensibles ante tal tóxico favoreciendo aquellos organismos resistentes ("Pollution-induced community tolerance"). De esta manera, la tolerancia de las poblaciones se verá aumentada gracias al mantenimiento de alelos resistentes en la población (Blanck y Dahl 1996). Los organismos aislados de aguas continentales tienen más "acceso" a contaminantes ambientales derivados de las actividades humanas, por lo que cabe esperar que las poblaciones presenten una mayor capacidad adaptiva. De la misma manera, aunque en menor magnitud, los organismos de zonas costeras también se exponen más habitualmente a esta contaminación, mostrando así, capacidades de adaptación intermedias. En el caso de los organismos procedentes de mar abierto o dinoflagelados simbióticos de los corales, en los que su contacto previo con cualquier agente contaminante parece altamente improbable, la capacidad de adaptación fue reducida (Tabla III). Cuando el agente selectivo fue la temperatura, cabría esperar que los organismos con capacidad de adaptación a temperaturas más altas fueran aquellos procedentes de aguas continentales, seguidos de los aislados en aguas costeras, puesto que el rango de temperaturas que experimentan al año es mucho mayor, por lo que los organismos deberían estar seleccionados para sobrevivir ante tales fluctuaciones (Tabla III).

Aparte de la capacidad diferencial de adaptación, el experimento de Ratchet permite investigar si la capacidad de adaptación se garantizó por medio de procedimientos genéticos o de lo contrario, surgió como consecuencia de un proceso de

aclimatación fisiológica. En todos los casos se observó que la adaptación a la máxima presión selectiva se consiguió por medio de mecanismos genéticos. El tiempo necesario para la adquisición de la adaptación fue distinto entre réplicas de una misma especie (Tabla III). Asimismo, la tasa de crecimiento de las poblaciones derivadas fueron menores que aquéllas de los organismos ancestrales. Estas respuestas confirman resultados previos que apuntaban hacia el origen genético de estos resistentes (García-Villada *et al.* 2004; Marvá *et al.* 2010).

La biogeografía del fitoplancton está determinada por factores ambientales locales que seleccionan a las especies sobre la base de su potencial óptimo de crecimiento. Nuestros datos, demuestran una capacidad de adaptación diferencial inter-específica que puede derivar no sólo en un cambio en la estructura de las comunidades fitoplanctónicas, como ya han observado distintos modelos experimentales (Litchman *et al.* 2006), sino en una reducción en la diversidad de especies. Obviamente, no se puede hacer una predicción real sin tener en cuenta el resto de componentes involucrados en el desarrollo de las comunidades, en el que la competición "in situ" de especies sería un factor muy importante. La composición de la comunidad fitoplanctónica afecta profundamente los patrones de ciclo de nutrientes, la dinámica de la red trófica y la producción primaria neta en el océano (Doney *et al.* 2002). Por ello, las predicciones sobre la estructura futura de la comunidad fitoplanctónica resultan cruciales para determinar la respuesta de los océanos ante el cambio ambiental futuro. El diseño experimental propuesto, puede aportar una primera aproximación para explorar la respuesta inter-específica ante un cambio ambiental como el calentamiento global.

Hasta este punto se ha considerado la posibilidad de adaptación de los organismos ante ambientes selectivos diversos. Y si esta adaptación es posible, se ha determinado si puede asegurarse por medio de una respuesta fisiológica o gracias a la adaptación genética. Pero la adaptación genética no es la única fuerza que conduce el cambio evolutivo (Gould 2002). Si todo cambio evolutivo en las poblaciones fuera fruto de la adaptación, el valor adaptativo de los individuos podría determinarse según los

caracteres fenotípicos que presente cada individuo concreto. Sin embargo, existen otra serie de factores que contribuyen en mayor o menor medida al fenómeno de la evolución, como son el azar o la contingencia histórica. Para distinguir la contribución relativa de cada uno de estos factores al proceso evolutivo se estudió la evolución concreta de la cianobacteria *Microcystis aeruginosa* ante cambios ambientales bruscos, el aumento de temperatura y eutrofización. Con este análisis, se pretendía comprobar distintas hipótesis: (1) la evolución adaptativa es el principal conductor de la evolución de caracteres ligados a la *fitness* o eficacia biológica (como ha sido previamente demostrado en trabajos anteriores de Travisano *et al.* 1995a y Flores-Moya *et al.* 2008); (2) el azar y la contingencia histórica son los principales motores del cambio evolutivo de caracteres con poco valor adaptativo y menor trascendencia para la eficacia biológica. Por ello, los caracteres elegidos para su estudio fueron la tasa de crecimiento (principal componente de la *fitness* -Fisher 1930-) y la producción de toxina, cuya correlación con la *fitness* es baja (López-Rodas *et al.* 2006). Así, los resultados demostraron que, en el caso de la tasa de crecimiento, su evolución se debió fundamentalmente a un proceso adaptativo, aunque el azar también contribuyó pero en menor medida. Por el contrario, en el caso de la producción de toxina, su evolución se debió fundamentalmente a un fenómeno azaroso, permaneciendo así mismo la huella de la historia. En general, caracteres débilmente asociados a la *fitness*, presentan heredabilidades altas, como es el caso de la toxina (López-Rodas *et al.* 2006), indicando que no poseen gran importancia evolutiva.

Mediante el desarrollo de este tipo de experimentos encaminados a determinar el papel de los distintos contribuyentes en el proceso evolutivo, se pueden conseguir distintos objetivos: (1) Por un lado, si la adaptación es el principal conductor del cambio evolutivo, podrían realizarse predicciones sobre el comportamiento de diversas especies fitoplanctónicas en distintos escenarios de cambio ambiental. De esta manera, de los resultados de este estudio, se podría predecir un aumento de los *blooms* de cianobacterias con el aumento de la temperatura y eutrofización. Por el contrario, si el azar o la historia influyen en mayor grado que el componente adaptativo, la dirección de la evolución es impredecible, como es el caso de la toxina en este estudio. (2) Por otro

lado, el resultado de estos estudios podría arrojar información sobre la importancia evolutiva y ecológica de determinados caracteres cuya función no está aún determinada. Como ejemplo, podríamos citar la gran controversia en la función del fenómeno de bioluminiscencia que presentan determinados dinoflagelados (Young 1983, Abrahams y Townshend 1993). Podría sugerirse que su presencia en las poblaciones fuera meramente fortuita y debida únicamente a efectos del azar y contingencia histórica, y por tanto, la búsqueda de su función ecológica no tuviera demasiado sentido.

En este punto de la discusión conviene introducir un pequeño comentario relativo a las limitaciones del diseño experimental en experimentos de evolución. Como expusieron Lenski y Travisano en uno de sus manuscritos sobre evolución experimental (1994): "*We acknowledge the severe limitations inherent in our study of evolutionary dynamics. Foremost among these are the shot time span (...) and the simple environment (which ignores the complexity and changeability of nature). The former limitation reflects our lack of access to better machines for time travel, and the latter our desire as experimentalist to keep things simple enough that we may understand the results. In terms of these limitations, we are certainly studying the tempo and mode of microevolution*". Asimismo, los estudios realizados en esta tesis mantienen cultivos puros, para el estudio de cada especie fitoplanctónica aislada de otro tipo de otro tipo de organismos. Obviamente, el comportamiento observado en estos cultivos mantenidos en condiciones estériles se simplifica, debido a la ausencia de interacción entre miembros de las poblaciones naturales con los que los organismos han co-evolucionado durante millones de años. Pero todo diseño experimental presenta limitaciones, y a veces se debe recurrir a la simplificación de la realidad para poder comprender procesos más complejos.

Los primeros capítulos de esta tesis han abordado el estudio de la repuesta evolutiva del fitoplancton ante una gran diversidad de condiciones ambientales. Por ello, para culminar este trabajo de tesis, se consideró interesante la inclusión de un estudio fisiológico. Como se ha ido comentando, si la presión selectiva aplicada en un

ambiente concreto no supera los límites de tolerancia fisiológica de los organismos, estos serán capaces de responder ante tal cambio por medio de la modulación de la expresión de sus genes (Bradshaw y Hardwick 1989). El estudio de la modificación de distintas capacidades fisiológicas del fitoplancton ante determinados cambios ambientales es importante, considerando la gran influencia de estos microorganismos como productores primarios y sobre los ciclos geoquímicos, y en general, en el mantenimiento del equilibrio terrestre. En concreto, el organismo estudiado en este último trabajo, el cocolitofórido *Emiliania huxleyi,* juega un papel muy importante en la regulación del ciclo del carbono global (Archer *et al.* 2000) debido a la producción de placas de carbonato cálcico, mediante el mecanismo de calcificación, y a su relevancia en la producción primaria del Atlántico Norte (Smyth 2004). Por ello, el efecto que cualquier alteración en el medio pudiera ocasionar sobre algunos de estos procesos, podría causar consecuencias importantes en el mantenimiento del ciclo del carbono en grandes regiones oceánicas.

Durante los últimos años, un gran número de estudios se han centrado en las consecuencias del aumento de CO_2 atmosférico sobre distintas funciones fisiológicas de los organismos. En concreto, gran parte de la investigación en esta línea está encaminada al efecto de la acidificación oceánica sobre el proceso de calcificación. Sin embargo, la variación en un determinado parámetro ambiental no ocurre independientemente en el océano sino que habitualmente repercute en el resto de variables. En los ambientes naturales los organismos están sujetos simultáneamente o dentro de un espacio de tiempo corto a más de un tipo de estrés. Consecuentemente, es importante analizar distintos parámetros ambientales al mismo tiempo para estudiar su efecto combinado y poder obtener una visión más realista del proceso. Así, se decidió estudiar el efecto combinado de dos variables, los niveles de CO_2 y la disponibilidad de nutrientes, sobre la fisiología del cocolitofórido *Emiliania huxleyi*. Para ello, se seleccionaron tres concentraciones de CO_2 atmosférico distintas, simulando distintos escenarios (pre-industrial, presente y futuro), y se cultivaron en condiciones de baja disponibilidad de fósforo y nitrato. En concreto se caracterizó la variación en su capacidad calcificadora y la modificación en la actividad de dos enzimas implicadas

estrechamente en la asimilación de nitrato y fosfato: la nitrato reductasa (NR) y la fosfatasa alcalina (AP).

La respuesta del fitoplancton y, en general, de distintos organismos, ante el aumento de la concentración atmosférica de CO_2 es variada. La afectación de la fisiología de cada especie, fotosíntesis y crecimiento, dependerá de sus mecanismos de captación de carbono inorgánico disuelto y la afinidad selectiva por CO_2 o HCO_3^{2-}, así como de su capacidad de reacción ante cualquier modificación del sistema (Rost *et al.* 2003; Hurd *et al.* 2009). Asimismo, los organismos calcificadores, requieren del proceso de calcificación en ciertas fases de su ciclo vital. No solo fitoplancton, sino otros organismos superiores como corales, foraminíferos bénticos, moluscos, equinodermos y crustáceos (Raven *et al.* 2005; Kleypas *et al.* 2006; Tyrell 2008; Doney *et al.* 2009) verán alterada su capacidad para producir estructuras calcáreas ante el aumento de CO_2 atmosférico. Esto es debido, tanto a la disminución del ión [CO_3^{-2}] en la columna de agua, esencial para la calcificación, como al desplazamiento hacia la superficie de los horizontes de saturación del carbonato cálcico, derivando en una mayor disolución de sus dos formas biológicas, la calcita y el aragonito (reviewed by Kleypas 2006; Dickson 2010). Aunque la tendencia hacia la disminución de las estructuras calcáreas en estos organismos, fruto de la acidificación oceánica, parece clara, distintos trabajos en el cocolitofórido *Emiliania huxleyi* presentan resultados diversos (Riebesell *et al.* 2000, Sciandra *et al.* 2003; Engel *et al.* 2005; Zondervan 2007; Iglesias-Rodriguez *et al.* 2008; Barcelos e Ramos *et al.* 2009; Langer *et al.* 2009; Shi *et al* 2009; Müller *et al.* 2010). Esta variabilidad podría ser atribuida a diferencias de respuesta según cepas (Langer *et al.* 2006).

Por otro lado, la disponibilidad de nutrientes afecta también en gran medida distintos procesos fisiológicos del fitoplancton. Los organismos requieren de distintos nutrientes para el desarrollo de su metabolismo, siendo el fósforo y nitrógeno dos elementos esenciales. La disponibilidad del nitrógeno y fósforo disuelto en aguas oceánicas también influencia la composición y variedad de especies en las poblaciones

naturales de fitoplancton (Egge 1994). Para luchar contra alteraciones en la biodisponibilidad de nutrientes, los organismos cuentan con distintas estrategias para favorecer su *uptake* (captación) y su asimilación, aumentando asimismo la expresión de distintas actividades enzimáticas involucradas. Se ha visto que el cocolitofórido Emiliania huxleyi puede aprovechar distintas fuentes de nitrógeno orgánico disuelto (Antia 1975; Palenik y Henson 1997) gracias al aumento de la expresión de distintos genes relacionados con el metabolismo del nitrógeno (Song y Ward 2007; Dyrman 2006b). Esta característica, junto con su excepcional capacidad para la adquisición de fosfato de fuentes orgánicas, en situaciones de limitación por fósforo inorgánico, a través de la enzima fosfatasa alcalina, (Riegman 2000; Dyhrman y Palenik 2003; Dyrhman *et al.* 2006a, Landry *et al.* 2006), confiere a esta especie una ventaja competitiva en ambientes de estrés por nutrientes (Lessard 2005). Por ello, los blooms de esta especie se ven favorecidos en determinadas regiones oligotróficas como zonas del Atlántico Norte (Tyrrel and Tailor 1996). Además, estas características, determinan en gran medida el orden de las distintas especies en la sucesión de los blooms fitoplanctónicos (Marañon 1996; Litchman 2006). Por otro lado, la Nitrato reductasa, es una enzima altamente involucrada en el metabolismo del nitrógeno en plantas. Esta enzima cataliza la reducción de nitrato a nitrito, el primer paso para la asimilación del nitrógeno en las células de las plantas (Solomon 1990) y requiere de la presencia de nitrato para su expresión (Kaffes 2010). Ante concentraciones de nitrato suficientes y no siendo el amonio la fuente predominante, la asimilación de nitrógeno se consigue fundamentalmente a través de la actuación de la nitrato reductasa, dando menor prioridad a otras vías de asimilación de nitrógeno orgánico (Bruhn 2010). Además, la distinta disponibilidad de nutrientes afecta el ratio PIC:POC en poblaciones de *Emiliania huxleyi*, como se ha visto en este estudio y en otros estudios previos (Riegman 2000). Asimismo, ante condiciones de limitación por fosfato, se observó la activación no solo genes relacionados con la asimilación del fosfato, sino también aquellos reslacionados con el mecanismo de calcificación o la homeostasis del calcio (Dyrhman 2006b).

Por ello, con este estudio se perseguían dos objetivos: (1) aportar nuevos datos sobre la respuesta del mecanismo de calcificación de esta especie y la alteración de los

ratios PIC:POC, en este caso añadiendo "nutrientes" como una nueva fuente de variación e (2) investigar la capacidad asimiladora de nutrientes de esta especie en un escenario de acidificación oceánica mediante el estudio de la modificación de la actividad de las enzimas AP y NR.

Como ya se vio en el capítulo 5, los resultados obtenidos mostraron que la respuesta del mecanismo de calcificación de esta cepa de *Emiliania huxleyi* ante el aumento de CO_2 atmosférico es muy variada. Cuando nitrato y fosfato no se encontraban en condiciones limitantes, la calcificación de esta especie tendió a aumentar con el incremento del CO_2 atmosférico, como ya vieron trabajos anteriores con la misma cepa (Iglesias-Rodríguez 2008). No obstante, en condiciones de limitación por fosfato o nitrato, no se siguió el mismo patrón, presentándose respuestas diversas según la concentración de CO_2 atmosférica. El PIC (carbono inorgánico particulado) por célula fue mayor en los cultivos bajo nutrientes limitantes ante las dos primeras condiciones de CO_2 (255 y 527 ppm), como ya se había observado en estudios previos con esta especie (Paasche 1994, 1998; Rieman 2000). Sin embargo, no se observó tal diferencia ante concentraciones de CO_2 elevadas (1205 ppm). El ratio PIC:POC presentó asimismo patrones distintos con el aumento de CO_2 atmosférico en función de la disponibilidad de nutrientes. Sin embargo, no superó en ningún caso el límite de 1.5, por encima del cual se considera que los blooms de estos organismos pasarían a ser fuente de CO_2 a la atmósfera, en lugar de sumidero.

La actividad de las enzimas nitrato reductasa y fosfatasa alcalina se vio asimismo alterada con el aumento de CO_2 atmosférico. Pero, como consideró Berges (1997), es importante definir la interpretación de la actividad de la fosfatasa alcalina y la nitrato reductasa en este estudio. La determinación de la actividad se realiza mediante un estudio "in vitro" que requiere condiciones fijas de pH, temperatura y concentración de sustrato. La actividad de las enzimas es medida ante concentraciones de sustrato "saturantes", lo que es definido como un "ensayo V_{max}". Ante estas condiciones, la actividad se considera como la expresión de la cantidad de enzima (Rossomando 1990). Como ya se vio en el capítulo 5, la enzima fosfatasa alcalina disminuyó su actividad

ante condiciones de pH reducidas, como las presentadas en el escenario de CO_2 más elevado (1205 ppm). Este hecho, deriva posiblemente, de la naturaleza alcalina de esta enzima. En el caso de la nitrato reductasa, al contrario, la actividad de esta enzima, en presencia de nitrato en el medio, aumentó con el aumento de CO_2 atmosférico. Este hecho podría estar relacionado con la estrecha relación entre los mecanismos de asimilación de carbono y nitrógeno, y la observada modulación en paralelo de la nitrato reductasa con el flujo electónico durante la fotosíntesis (Beardall 1998; Sherameti 2002). Esta relación es también apreciada en este estudio si se considera el aumento del carbono orgánico particulado (POC), en los cultivos bajo limitación por nitrógeno, asociado al incremento de CO_2 atmosférico.

Con estos resultados, se podría considerar que la ventaja competitiva del cocolitofórido *Emiliania huxleyi* en las poblaciones naturales y en el desarrollo de los blooms podría verse alterada en determinadas condiciones de acidificación oceánica y limitación por nutrientes. Pero por supuesto, no se puede establecer una conclusión final sin tener en cuenta la respuesta del resto de especies fitoplanctónicas ante las mismas variables, así como la interacción con otras variables del sistema. Cambios en la asimilación de nutrientes tanto en *E.huxleyi* como en otras especies fitoplanctónicas pueden dar lugar a alteraciones en la dinámica de las poblaciones así como la distribución espacial de especies, conduciendo a alteraciones en los ciclos biogeoquímicos, y en general, en la dinámica global del clima.

En resumen, todos estos trabajos han estudiado el efecto de distintos fenómenos de cambio global sobre la evolución del fitoplancton y su fisiología. Distintas especies se adaptarán de manera diferente presentando tolerancias y capacidades de adaptación diversas. Asimismo, este último estudio fisiológico nos muestra el cambio en la fisiología de una especie concreta cuando esta es expuesta a la alteración de su medio. Por ello, se puede comprender como la interacción de los distintos organismos con el medio y su capacidad de respuesta ante condiciones ambientales alteradas, pueden determinar la capacidad de supervivencia y competición del mismo en el ecosistema. De esta manera, se alterará la distribución espacial de especies y la estructura de las

comunidades afectando, en última instancia a la transferencia de energía a niveles tróficos superiores y al destino biogeoquímico del carbono y nutrientes.

Tabla I: Tasas de mutación (μ) del paso de sensibilidad a resistencia de distintas especies de microalgas y cianobacterias frente a distintas aguas extremas naturales. La tasa de mutación se expresa como número de mutantes por división celular. q= frecuencia mutante resistente (número de células resistentes por cada célula sensible).

Ambientes extremos					
Origen del agua	**Características agua**	**Microbiota**	**Tasa de mutación (μ)**	**Frecuencia mutante resistente (q)**	**Fuente**
Agrio Argentino	pH: 4.1 Nivel alto de metales	*K. raphidioides*[1] *Ulothrix* sp. [1] *Chlamydomonas* sp. [1] *Dictyosphaerium pulchellum* H. C. Wood[1] *Viridiella* sp. [1] *Spirogyra* sp. [2] *Fragillaria* sp. [3] *Euglena mutabilis* Schmitz[4]	**DcG1:** 5.4 x 10^{-7} **Ma3D:** no adaptación a ningun agua	18 x 10^{-7}	López-Rodas *et* al. 2010 (**INCLUIDO EN ESTA TESIS**)
Vulcano	pH 3.1 Niveles de Sulfídico: 1.84 ± 0.10 g L^{-1} Tª: 30.3 ± 0.5	*Chlamydomonas variabilis*[1] *Dictyosphaerium ehrenbergianum*[1] *Stephanodiscus sp.* [3] *Euglaena sp.* [4]	**Dc1M**: 4.7 x 10^{-7} **MaD:** no adaptación	No medido	López-Rodas *et* al. 2009 (**INCLUIDO EN ESTA TESIS**)
Aguas Agrias	pH: 2.5 Nivel alto de metales	*Nitzschia acicularis (Kützing) W. Smith*[3] *Navicula exigua Gregory*[3] *Fragilaria sp.*[3] *Streptophyta Spirogyra sp.*[5] *Chlorophyta Chlorella sp.*[1]*Oocystis borgei J.*[1] *Chlamydomonas sp.*[1]*Scenedesmus arcuatus Lemmermann*[1]*Euglena sp.*[4] *Trachelomonas granulosa Playfair*	**Dc1M**: 1.1 x 10^{-6}	12 x 10^{-7}	López-Rodas *et* al. 2008b (**INCLUIDO EN ESTA TESIS**)

Mynydd Parys Pond	pH: 2.4-2.7 Nivel alto de metales	*Spirogyra communis* (Hassall) Kützing[1] *Chlorella* sp.[1] *Chlamydomonas variabilis* P.A. Dangeard[1] *Chloroccocum infusionum* (Schrank) Meneghini[1] *Euglenophyta Euglena geniculata* (F. Schmitz) Dujardin[4] *Phacus longicauda* (Ehrenberg) Dujardin[4]	**Dc1M**: 1.6 x 10^{-6}	19 x 10^{-7}	López-Rodas *et* al. 2008c
Aguas geotermales	pH: 2.5-7	<u>Según pH y toxicidad</u> **pH 6.4-7**: alta diversidad de microalgas y cianobacterias. **pH 5.7-6.7**: baja diversidad microalgas y cianobacterias o solo microalgas dependiendo de la toxicidad del agua. **pH 2.5-3.2**: baja diversidad de clorofitas.	**Dc1M**: 1.4 x 10^{-6} - 1.5 x 10^{-5} **MaD:** 1.1 x 10^{-6} – 1.1 x 10^{-5}	**Dc1M**: 11 a 200 x 10^{-7} **MaD:** 14 a 120 x 10^{-7}	Costas *et* al. 2008
Río Tinto	pH: 1.7-2.5	Principalmente clorofitas (Amaral-Zettler *et* al. 2002)	**Dc D**: 1.38 10^{-6} **MaD:** no adaptación	15 x 10^{-7}	Costas *et* al. 2007
La Hedionda	Altos niveles de sulfuro	*Spirogyra sp.*[2] (la más abundante) *Oscillatoria*[6] *Navicula*[3] *Nitzschia*[3] *Achnanthes*[3]	**Sp_i**: 2.7 10^{-7}	No medido	Flores-Moya *et* al. 2005
Aznalcollar	Niveles altos de metales pesados y metaloides	No determinado	**Sc_i31M**: 2.12 10^{-5}	65 x 10^{-4}	Baos *et* al. 2002

Tabla II: Tasas de mutación (μ) del paso de sensibilidad a resistencia de distintas especies de microalgas y cianobacterias frente a distintos contaminantes antropogénicos a dosis letales. La tasa de mutación se expresa como número de mutantes por división celular.

Contaminantes antropogénicos			
***Dictyosphaerium chlorelloides* (Dc1M)**			
Sustancia	**Tasa de mutación (μ)**	**Frecuencia mutante resistente (q)**	**Fuente**
Simazina	**Dc1M**: 9.2 x 10^{-6}	30 x 10^{-6}	Marvá *et* al. 2010 (**INCLUIDO EN ESTA TESIS**)
Cromo	**Dc1M**: 1.77 x 10^{-6}	14 x 10^{-6}	Sánchez-Fortún *et* al. 2009 (**INCLUIDO EN ESTA TESIS**)
Formaldehido	**Dc1M** : 3.6 x 10^{-6}	77 x 10^{-4}	López-Rodas *et* al. 2008a (**INCLUIDO EN ESTA TESIS**)
TBT	**Dc1M** : 7.07 x 10^{-8}	3 x 10^{-4}	López-Rodas *et* al. 2010
TNT	**DcG1** :1.4 x 10^{-5}	59 x 10^{-4}	García – Villada *et* al. 2002
DCMU	**DcG1:** 2.1 x 10^{-6}	21 x 10^{-4}	Costas *et* al. 2001
Scenedesmus intermedius			
Simazina	**Sc_iM y Sc_iD**: 3 x 10^{-6}	11 a 28 x 10^{-6}	Marvá *et* al. 2010 (**INCLUIDO EN ESTA TESIS**)
Diquat	**Sc_iD**: 17.9 x 10^{-6}	83 x 10^{-6}	Marvá *et* al. 2010 (**INCLUIDO EN ESTA TESIS**)
Cloranfenicol	**Sc_iD**: 1.1 x 10^{-5}	3 x 10^{-3}	Sánchez-Fortún *et* al. 2009 (**INCLUIDO EN ESTA TESIS**)
TNT	**Sc_iFNMS1**: 8.2 x 10^{-6}	31 x 10^{-4}	López-Rodas *et* al. 2001
Microcystis aeruginosa			
Glifosate	**Ma3D**: 3.6 x 10^{-7} **Ma7D**: 3.1 x 10^{-7}	65 x 10^{-4}	López-Rodas *et* al. 2007
Sulfato de cobre	**MaM1**: 1.76 x 10^{-6}	15 x 10^{-4}	García-Villada *et* al. 2004
Pseudoanabaena sp.			
DCMU	2.4 x 10^{-6}	6 x 10^{-4}	López-Rodas *et* al. 2001
Eritromicina	2.1 x 10^{-6}	16 x 10^{-4}	
Dunaliella tertiolecta			
DCMU	3.6 x 10^{-6}	21 x 10^{-4}	López-Rodas *et* al. 2001

Tabla III. Máxima capacidad de adaptación de distintas especies fitoplanctónicas a diversas presiones selectivas (sulfato de cobre, temperatura y simacina) y número de generaciones requeridas para alcanzar esta adaptación (g). (-) no determinado; (x) la adaptación no se dio más allá por lo que no se experimentó ninguna generación.

Lugar de aislamiento	Cepa	Sulfato de cobre		Temperatura		Simacina	
		µM	g	°C	g	µM	g
AGUAS CONTINENTALES	*Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman	270	230	35	105	40.5	300
		270	253	35	135	40.5	300
		90	184	35	135	40.5	255
	Scenedesmus intermedius (1D)	270	230	-	-	-	-
		270	230	-	-	-	-
		270	230	-	-	-	-
	Scenedesmus intermedius (1A)	270	240	-	-	-	-
		270	240	-	-	-	-
		270	270	-	-	-	-
	Scenedesmus intermedius (Chodat)	-	-	40	180	13.5	300
		-	-	40	180	13.5	300
		-	-	40	195	13.5	315
	Microcystis aeruginosa (3D)	30	84	35	32	0.45	75
		10	48	35	32	0.45	120
		10	48	35	24	0.45	93
	Microcystis aeruginosa (6D)	10	36	35	32	0.45	90
		10	48	35	32	0.45	105
		10	36	35	40	0.45	98
	Microcystis aeruginosa (7D)	10	48	35	53	0.45	76
		10	48	35	60	0.45	76
		10	36	35	60	0.45	75
OCÉANO ABIERTO	*Emiliania huxleyi* (CCMP371)	-	-	22	X	0.15	36
		-	-	22	X	0.15	36
		-	-	22	X	0.15	32
	Emiliania huxleyi (CCMP372)	-	-	22	X	0.15	32
		-	-	22	X	0.15	32
		-	-	22	X	0.15	36
	Emiliania huxleyi (CCMP373)	-	-	22	X	0.15	40
		-	-	22	X	0.15	36
		-	-	22	X	-	X

	Isochrysis galbana	-	-	35	60	0.15	40
		-	-	35	50	0.15	45
		-	-	35	50	0.15	40
	Monochrysis lutheri	-	-	22	X	0.15	45
		-	-	22	X	0.15	45
		-	-	22	X	0.15	40
ALGAS SIMBIÓTICAS DE LOS CORALES	*Symbiodinium* (CCMP2429)	-	-	30	65	-	-
		-	-	30	55	-	-
		-	-	30	60	-	-
	Symbiodinium (CCMP2433)	-	-	30	60	-	-
		-	-	30	70	-	-
		-	-	30	65	-	-
AGUAS DE ZONAS COSTERAS	*Tetraselmis suecica*	-	-	35	105	1.5	150
		-	-	35	105	1.5	165
		-	-	35	135	1.5	150
	Phaedactilum tricornutum	-	-	22	X	0.45	150
		-	-	22	X	0.45	165
		-	-	22	x	0.45	150
	Prorocentrum triestinum	-	-	30	25	-	-
		-	-	30	25	-	-
		-	-	30	30	-	-
	Nitzschia chlosterum	-	-	30	20	-	-
		-	-	30	30	-	-
		-	-	30	20	-	-
	Navícula sp.	-	-	30	27	-	-
		-	-	30	34	-	-
		-	-	30	20	-	-

LEYENDA DE LAS TABLAS

- Especie:

  Dc: *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman[1]

  Sc_i: *Scenedesmus intermedius* Chodat[1]

  Ma: *Microcystis aeruginosa* (Kützing) Lemmermann[6]

  Sp_i: *Spirogira insignis* (Hassal) Kützing[2]

  Dt: *Dunaliella tertiolecta* Butcher[1]

  Ps: *Pseudoanabaena sp.*[3]

- Grupo taxonómico:

  [1]Chlorophyta [2]Charophyta [3]Bacillariophyta

  [4]Euglenozoa [5]Streptophyta [6]Cianophyta

- Lugar de aislamiento:

  Dc1M: Lago prístino y ligeramente alcalino de alta montaña en el Parque Nacional de Sierra Nevada (España).

  DcD: Lago prístino de aguas no ácidas del Parque Nacional de Doñana (España).

  DcG1: Lago Parque Nacional de Sierra Nevada (España) donde no había existido contacto anterior con herbicidas.

  Ma(3, 6)D: Lago de aguas no ácidas del Parque Nacional de Doñana (España).

  MaM1: Embalse de abastecimiento (La Minilla) en Sevilla (España) que nunca había sido tratado con alguicidas.

  Sc$_i$31M: embalse de Segovia.

  Sc$_i$M: Embalse de Entreka en el desierto de Sahel (Mauritania) donde los organismos no han estado nunca expuestos a un herbicida.

  Sc$_i$D: Lago en el Parque Nacional de Doñana (España), expuesto a veces a residuos de herbicidas procedentes de áreas agrícolas cercanas.

VIII. CONCLUSIONES

1. Los mecanismos de adaptación de microalgas y cianobacterias a ambientes extremos dependen del grado de toxicidad de cada ambiente en concreto y si supera o no los límites de tolerancia fisiológica. Los mecanismos adaptativos comprenden la adaptación fisiológica, la adaptación genética, o la incapacidad de adaptación en último término si la toxicidad es extremadamente elevada. Dicho límite varía de unos organismos a otros.

2. Los organismos fotosintéticos del plancton son capaces de adaptarse genéticamente a dosis letales de contaminantes de origen antropogénico mediante mutaciones espontáneas, al azar, antes de entrar en contacto con el agente selectivo.

3. Las tasas de mutación varían entre 10^{-5} y 10^{-8} mutaciones por célula y generación, observándose una gran diversidad entre especies, cepas y contaminantes. Los resultados indican que estas tasas de mutación podrían variar en función de los genes analizados. A pesar del "coste de la adaptación" la proporción de individuos resistentes mantenidos en la población es suficiente para asegurar el mantenimiento y la supervivencia de las poblaciones ante los cambios ambientales.

4. Los organismos fitoplanctónicos del plancton presentan una capacidad de adaptación diferencial en función del grupo taxonómico al que pertenecen. Este hecho podría dar lugar a alguna alteración futura en la estructura de sus poblaciones.

5. La cianobacteria *Microcystis aeruginos*a incrementa su tasa de crecimiento cuando aumenta la temperatura y los nutrientes, previstos en un escenario de cambio global. Sin embargo, el carácter de producción de toxina parece determinado por mecanismos de azar y contingencia histórica.

6. El cocolitofórido *Emiliania huxleyi* altera su proceso de calcificación y la actividad de la nitrato reductasa y fosfatasa alcalina ante condiciones previstas del cambio global como es el aumento de CO_2 atmosférico y la variación en la disponibilidad de nutrientes. Las consecuencias de este hecho podrían alterar el ciclo global del carbono así como la capacidad competitiva de esta especie.

VIII. CONCLUSIONS

1. Cyanobacterial and microalgal mechanisms to adapt to extreme environments depend on the degree of toxicity of each specific environment and whether it exceeds the limits of physiological tolerance. The adaptive mechanisms include physiological adaptation, genetic adaptation and the incapacity to adapt if toxicity is too high. Such limits vary between organisms.

2. Photosynthetic planktonic organisms are able to adapt to lethal doses of contaminants from anthropogenic origin through spontaneous mutations which occurred randomly, prior to the toxic substance exposure.

3. Mutation rates vary between 10^{-5} y 10^{-8} mutations per cell division, showing interspecific and inter-strain variability and variation depending on toxic substances. The results show that these mutation rates could also vary depending on the analyzed gene. In spite of the "cost of adaptation", the proportion of individuals that are kept in the population after each generation is enough to maintain the genetic variability to ensure population survival after a sudden environmental change.

4. Phytoplanktonic organisms show different capacities for adaptation depending on their taxonomic group. Sudden environmental change could lead to an alteration of population structure and dynamics.

5. *Microcystis aeruginosa* evolved to a higher growth rate when it was exposed to increased temperature and nutrients. However, toxin production seems to be determined by chance and history mechanisms.

6. *Emiliania huxleyi* calcification as well as nitrate reductase and alkaline phosphatase activity are altered by exposure to increasing CO_2 and variation in

nutrient availability. This response could lead to alterations in global carbon cycle and competitive advantage of this microorganism.

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